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Altered protein and fatty acid composition of porcine follicular fluid due to a high fibre diet and the subsequent effects on oocyte maturation

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Declaration

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed and planned by myself in collaboration with my supervisors Prof. Cheryl J. Ashworth, Dr. Andrew C. Gill and Dr. Tom G. McEvoy. No part of this thesis has been or will be submitted for any other degree, diploma or qualification.

Selene Jarrett

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Carrying out a proteomic and metabolomic project was a challenge and I would not have been able to do it without the guidance of Dr Andrew Gill and Dr Dominic Kurian as well as a supportive network in the lab, courtesy of individuals like Charis Hogg, Barry Bradford, Sze Ying and Sam Eaton. I am also very appreciative of all the technical and academic advice given to me by my thesis committee members, Dr Jo Stevens and Dr Xavier Donadue and the statistical guidance provided by Dr Darren Shaw and Dr Helen Brown. Also, thank you Dr Elizabeth Ferguson and Dryden Farm for collecting the samples; without them the project would have never come to fruition. I am also grateful to Dr Tom McEvoy for all his advice regarding the *in vitro* maturation experiments and for lending me all the equipment that was required.

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Abstract

Background

Ovarian follicular fluid serves as the microenvironment for a maturing oocyte prior to ovulation. Previous studies have shown that gilts fed a high fibre (HF) diet before ovulation have improved fertility compared to gilts fed a control (C) diet, including a higher proportion of metaphase II oocytes following *in vitro* maturation (IVM).

Hypothesis

The molecular composition of porcine follicular fluid (pFF) was altered by the diet and that these alterations conferred the fertility benefits.

Aims

The aim of this study was to compare the protein composition of pFF from pigs fed a control diet with pFF of pigs fed a high fibre diet, to identify whether a high fibre diet fed to pigs during their oestrous cycle altered the composition of pFF. Additionally, the pFF of fertile animals was compared with the pFF of non-fertile animals to identify whether pFF composition was associated with fertility; fertile animals produced an embryo following *in vitro* fertilisation (IVF). Differences in the molecular composition were to be used to ascertain the potential underlying mechanism(s) involved in dietary induced improvements to oocyte maturation.

Results

The protein composition of pooled pFF from 12 HF-pigs and 12 C-pigs was compared by liquid chromatography tandem mass spectrometry (LC-MS/MS). Additionally, within each dietary group, the composition of pooled pFF from pigs whose oocytes produced blastocysts following *in vitro* fertilisation (C-Bl and HF-Bl) was compared with pFF from pigs whose oocytes did not produce blastocysts (C-No and HF-No respectively; n=6 per group). These proteomic analyses identified differentially expressed proteins, associated with several canonical pathways including acute phase

response signalling, complement system and LXR/RXR activation, as determined by Ingenuity Pathway Analysis.

Quantitative western blots revealed the differential expression of candidates associated with these canonical pathways. Plasminogen expression was lower ($P \leq 0.05$) in pFF of HF-pigs compared to pFF of C-pigs. In pFF from C-Bl gilts, apolipoprotein A4 ($P \leq 0.01$) and apolipoprotein M ($P \leq 0.05$) expression were higher compared to pFF from C-No gilts. Plasmin expression was lower ($P \leq 0.05$) in pFF from HF-Bl gilts compared to pFF from C-Bl gilts.

Due to the interest in the differentially expressed apolipoproteins (involved in cholesterol and lipid efflux), a targeted metabolomic analysis was carried out to measure the concentration of nine fatty acids (FAs) in pFF of individual pigs in C-No, C-Bl, HF-No, HF-Bl groups (n=6 per group); adrenic, arachadonic, arachidic, dihomo-γ-linolenic, docosapentaenoic, erucic, linoleic, palmitoleic and oleic acids were measured by LC-MS/MS. The analysis revealed the lower concentration of linoleic acid (LA, $p \leq 0.05$) and higher concentration of erucic acid ($P \leq 0.05$) in HF-pFF compared to C-pFF.

Following the results of the targeted metabolomic analysis, cumulus-oocytecomplexes (COCs) were matured in TCM 199 medium supplemented with 0 (No-LA), 50, 100 or 200 µM LA for 44 hours (n = 320 per treatment). COC diameters were measured and the COCs were categorised into "full", "partial" or "no" expansion. COCs were denuded, fixed and stained to determine their stage of maturation. IVM with 200 µM LA resulted in the reduced diameter of COCs ($p \leq 0.01$), fewer COCs with full cumulus expansion ($p \leq 0.05$) and fewer metaphase II oocytes ($p \leq 0.05$).

Discussion

Plasminogen is the precursor to plasmin, a proteolytic enzyme involved in weakening the follicular wall prior to ovulation. The lower expression of plasminogen and plasmin in pFF of high fibre pigs implies a delay in the accumulation of the inflammatory proteins required for ovulation. The delay in ovulation can result in the

lengthening of the oocyte maturation process, leading to more mature oocytes, as observed in the previous studies. A disruption in the expression of apolipoproteins may also occur in high fibre-fed pigs. The increase in apolipoproteins associated with blastocyst development was only observed with pFF of control pigs but not high fibre pigs. An alteration in lipid homeostasis in the high fibre pigs could potentially affect oocyte energy consumption. LA concentration was also lower in pFF of high fibre pigs. LA is an essential fatty acid, indicating that the difference in concentration is directly from the diet. The lower levels of LA can potentially be beneficial to oocyte maturation, which is substantiated by the negative effects of a high LA concentration on IVM of abattoir derived oocytes.

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Lay Summary

Previous studies showed that pigs fed a high fibre diet before ovulation have improved fertility compared to pigs fed a control diet. Ovarian follicular fluid is a fluid that surrounds an egg cell as it matures in the ovary prior to ovulation. The aims of this study were to identify whether the high fibre diet altered the composition of proteins and fatty acids in follicular fluid and to identify whether these alterations were associated with improved fertility.

The aims of this study were to identify proteins that were present at different amounts between follicular fluids of control-fed pigs and high fibre-fed pigs, and between follicular fluids of fertile animals and non-fertile animals; fertile animals produced an embryo following *in vitro* fertilisation (IVF). Two of these proteins (plasminogen and plasmin) were confirmed as being lower in high fibre follicular fluids and are involved in preparing the ovarian tissue for ovulation, indicating a delay in ovulation and a lengthening in the maturation of the egg cell in the ovary. Another two proteins (apolipoprotein A4 and apolipoprotein M) which are involved in the regulation of fats, such as cholesterol, were higher in fluids of fertile control pigs compared to non-fertile control pigs, but not higher in fertile high fibre pigs compared to non-fertile high fibre pigs. This indicated an alteration in fat regulation in the high fibre pigs, which could potentially affect the energy consumption for the egg cell.

Due to an interest in fat regulation, the concentration of nine fatty acids (major components of fats) were measured. This identified the lower concentration of linoleic acid and higher concentration of erucic acid in fluids of high fibre fed pigs compared to fluid of control fed pigs. Cell culture experiments confirmed the detrimental effects of a high concentration of linoleic acid on the development of maturing egg cells. Therefore, the lower levels of linoleic acid due to the diet can potentially be beneficial to the maturation of the egg cells.

Conferences, Seminars and Meetings

Jarrett S, Gill AC, McEvoy TG and Ashworth CJ (January 2018) High linoleic acid concentration in porcine follicular fluid and maturation medium affects oocyte maturation and cumulus expansion. Fertility 2018 Conference Handbook [*Oral Presentation; Liverpool, United Kingdom*]

Jarrett S, Gill AC, Ferguson EM, McEvoy TG and Ashworth CJ Associations between pre-mating diet, follicular fluid linoleic acid concentrations and female fertility. AHDB 2017 Livestock PhD Seminar [*Oral Presentation; Stratford upon Avon, United Kingdom*]

Jarrett S, Gill AC, Ferguson EM and Ashworth CJ (July 2017) Metabolomics of porcine follicular fluid reveal the potential role of linoleic acid and erucic acid in oocyte maturation. Little Embryos Do Make Big Decisions Symposium Poster 5, P17–18 [*Poster Presentation; Southampton, United Kingdom*]

Jarrett S, Gill, AC, Kurian D, Ferguson EM and Ashworth CJ (June 2017) Proteomics and metabolomics of porcine follicular fluid reveal differential expression of apolipoproteins, plasminogen and fatty acids associated with pre-mating diet and fertility. 10th International Conference on Pig Reproduction Proceedings P22–23 [*Oral Presentation; Colombia, Missouri, United States*]

Jarrett S, McEvoy TG, Gill AC and Ashworth CJ (May 2017) Proteomics and metabolomics of porcine follicular fluid reveals the potential role of linoleic acid in oocyte *in vitro* maturation. The Roslin Institute Postgraduate Research Student Day P7 Abstract T14 [*Oral Presentation; Edinburgh, United Kingdom*]

Jarrett S, Ferguson EM, Kurian D, Gill AC and Ashworth CJ (January 2017) Proteomic analysis on of porcine follicular fluid reveals the differential expression of apolipoproteins and plasminogen associated with pre-mating diet and later fertility. Fertility 2017 Conference Handbook P137 Abstract 152 [*Poster Presentation; Edinburgh, United Kingdom*]

Jarrett S, Gill AC, Kurian D, Ferguson EM and Ashworth CJ (November 2016) Apolipoproteins and plasminogen expression in porcine ovarian follicular fluid associated with diet and fertility. AHDB 2016 Livestock PhD Seminar [*Poster Presentation; Stratford upon Avon, United Kingdom*]

Jarrett S (June 2016) Impact of nutrition on ovarian follicular fluid: a key to improving fertility and litter size in pigs? The Roslin Institute Friday Seminar [*Oral Presentation; Edinburgh, United Kingdom*]

Jarrett S, Ferguson EM, Kurian D, Gill AC and Ashworth CJ (April 2016) Differential proteomic profiles of porcine follicular fluid associated with a high fibre diet and later fertility. The Roslin Institute Postgraduate Research Student Day P60 Abstract 308 [*Poster Presentation; Edinburgh, United Kingdom*]

Jarrett S, Ferguson EM, Kurian D, Gill AC and Ashworth CJ (January 2016) Altered protein composition of porcine follicular fluid due to a high-fibre diet and the potential for optimisation of *in vitro* media. Reproduction, Fertility and Development P23 Abstract 81; 42nd Annual Conference of the International Embryo Transfer Society [*Poster Presentation; Louisville, Kentucky, United States*]

Jarrett S, Ferguson EM, Kurian D, Gill AC and Ashworth CJ (December 2015) The contribution of oocytes and follicular fluid to pig fertility. AHDB 2015 Livestock PhD Seminar [*Poster Presentation; Coventry, United Kingdom*]

Jarrett S, Ferguson EM, Kurian D, Gill AC and Ashworth CJ (April 2015) Differential proteomic profiles of porcine follicular fluid associated with a high fibre diet and later fertility. The Roslin Institute Postgraduate Research Student Day P47 Abstract 220 [*Poster Presentation; Edinburgh, United Kingdom*]

Jarrett S, Ferguson EM, Kurian D, Gill AC and Ashworth CJ (July 2015) Differential proteomic profiles of porcine follicular fluid associated with high fiber diet and later fertility. Reproduction Abstracts 2 P017 for Society for Reproduction

and Fertility Annual Conference 2015 P31 [*Poster Presentation; Oxford, United Kingdom*]

Jarrett S, Gill AC, Kurian D, Ferguson EM and Ashworth CJ (December 2014) The contribution of oocytes and follicular fluid to pig fertility. AHDB 2014 Livestock PhD Seminar [Oral *Presentation; Kenilworth, United Kingdom*]

Jarrett S, Gill AC, Kurian D, Hogg CO, Ferguson EM and Ashworth CJ (September 2014) Proteins in porcine follicular fluid as potential biomarkers for fertility. Reproduction Abstracts 1 P155 for 3rd World Congress on Reproductive Biology P54 [*Poster Presentation; Edinburgh, United Kingdom*]

Jarrett S, Gill AC, Walling G, Kurian D, Hogg CO, Ferguson EM and Ashworth CJ (April 2014) The contribution of oocytes and follicular fluid to pig fertility. The Roslin Institute Postgraduate Research Student Day P27 Abstract 117 [*Poster Presentation; Edinburgh, United Kingdom*]

Publications

Jarrett S and Ashworth CJ. The role of dietary fibre in pig production, with a particular emphasis on reproduction (*Journal of Animal Science and Biotechnology* **9** 59)

Jarrett S, Ferguson EM, Kurian D, McEvoy TG, Gill AC and Ashworth CJ. Proteomic and metabolomic analysis of porcine follicular fluid revealed the differential expression of apolipoproteins, plasmin, plasminogen and linoleic acid associated with a high fiber diet and later fertility (*Working Manuscript*)

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List of Abbreviations

1-D PAGE	One-dimensional polyacrylamide gel electrophoresis
2-D PAGE	Two-dimensional polyacrylamide gel electrophoresis
ACN	Acetonitrile
AcOH	Acetic acid
AHDB	Agriculture and Horticulture Development Board
Al(-)	Allantoic fluid negative control
All-C	All control group
All-HF	All high fibre group
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AU	Arbitrary units
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
C-Bl	Control-fed animals with blastocyst
CID	Collision-induced dissociation
Cl-No	Control-fed animals without blastocyst
CLA	Conjugated linoleic acid
CO_2	Carbon dioxide
COC	Cumulus-oocyte-complex
COH	Control ovarian hyperstimulation
COX	Cycloxegenase
CV	Coefficient of variation
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Deionised water
DGLA	Dihomo-γ-linolenic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
E_2	Oestradiol
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay

FF	Follicular fluid
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
FXR	Farnesoid X receptor
GC	Gas chromatography
GDF9	Growth differentiation factor 9
GnRH	Gonadotropin releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotropin
HCl	Hydrochloric acid
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF-Bl	High fibre-fed animals with blastocyst
hFF	Human follicular fluid
HF-No	High fibre-fed animals without blastocyst
HPG	Hypothalamic-pituitary-gonadal
IGF-I	Insulin growth factor I
IPA	Ingenuity Pathway Analysis
InStd	Internal standard
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
LC	Liquid chromatography
LDL	Low density lipoprotein
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LXR	Liver X receptor
MARS	Multi Affinity Removal System
MES	2-(N-morpholino)ethanesulfonic acid
MFA	Monounsaturated fatty acids
MI	Metaphase I
MII	Metaphase II

MOPS	3-(N-morpholino)propanesulfonic acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS-dH_2O	Mass spectrometry grade deionised water
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
$NaHPO_4$	Sodium phosphate
NAPDH	Nicotinamide adenine dinucleotide phosphate
NCSU-23	North Carolina State University Medium 23
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
P_4	Progesterone
PA	Parthenogenetic activation
PBS	Phosphate buffered saline
PC	Postcoitum
PCOS	Polycystic ovary syndrome
PFA	Paraformaldehyde
pFF	Porcine follicular fluid
Pl(+)	Plasma positive control
PPP	Pentose phosphate pathway
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFA	Saturated fatty acids
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TCM 199	Tissue Culture Medium 199
TFA	Trifluoroacetic acid
TGF	Transforming growth factor

1. Background and General Introduction

1.1. Introduction to Reproductive Biology of Female Pigs

1.1.1. Pig production and pig reproduction

In the agricultural industry, a routine method of assessing the efficiency of pig production is to measure the number of piglets weaned per sow per year (Koketsu *et al.*, 2017). The number of piglets weaned can be increased by enhancing the fertility of female pigs (Ferguson *et al.*, 2003). Therefore, an increased understanding of the strategies that influence porcine reproductive physiology can influence the production efficiencies of agricultural herds.

1.1.2. Reproductive tract and the ovary

Female reproduction in pigs begins with the ovary, the organ that houses one half of the germ cells required to produce a new organism. The porcine ovary is in contact with several ligaments of the oviduct, which is itself connected to the bicornuate uterus, cervix and vagina (**Figure 1.1a**). The ovary is surrounded by a fibrous tunica albuginea which is externally covered by cuboidal epithelial cells known as germinal superficial epithelium. The ovary is composed of an outer cortex and inner medulla; the cortex consists of dense connective tissue forming oocytes, while the medulla consists of loose connective tissue, blood vessels, lymphatics and nerves (Johnson, 2007). Pigs are polyovular species and can therefore ovulate multiple oocytes from both ovaries in an oestrous cycle (**Figure 1.1b**). Pigs can ovulate between 15–30 oocytes in a cycle but the average ovulation varies both between breeds and between sows and gilts (Cárdenas et al., 2002; Knox et al., 2003; Soede et al., 2011).

1.1.3. Pre-ovulatory ovarian follicle

Depending on factors such as timing of boar contact, nutritional status, body weight, season, breed, disease status, social environment and management practices, gilts usually reach puberty when they are between 6–8 months old (Kyriazakis and Whittemore, 2006). A mature ovarian follicle at the precipice of ovulation is referred to as a pre-ovulatory or Graafian follicle. It is characterised by its size (between 5–8

mm in pigs, depending on the maturity of the animal) and its large fluid-filled antrum which contains the mature oocyte, surrounded by specialised granulosa cells called cumulus cells (Bagg et al., 2007). The structure composed of the oocyte and the cumulus cells is referred to as the cumulus-oocyte-complex (COC). Additionally, the Graafian follicle is composed of proliferating granulosa cells, the basal lamina (specialised extracellular matrix), expanded thecal cells that form the theca interna (vascularised with thecal capillaries) and the theca externa layers, and the outer stromal cells (**Figure 1.1c**).

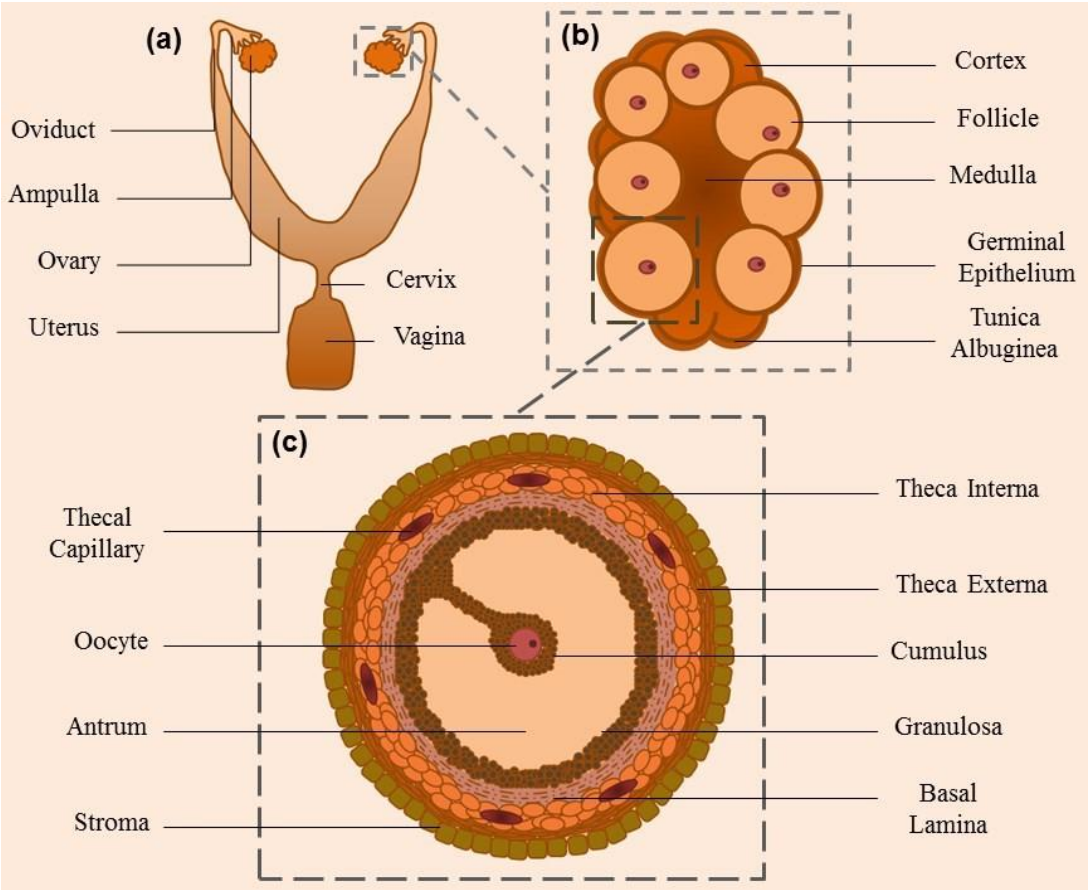


Figure 1.1. Schematic representation of the reproductive biology of the female pig.

The pig reproductive tract (a); the ovary is in contact with the ampulla, connecting it to the oviduct and uterus. Pigs have a bicornate uterus which is connected to the vagina via the cervix. Pigs are polyovular, which means that their ovaries have multiple Graafian follicles when they are at the praecipes of ovulation (b). These follicles are held within the cortical tissue of the ovary above the medulla and beneath the tunica albuginea and germinal superficial epithelium. The Graafian follicle (c) is characterised by the large follicular antrum which is filled with follicular fluid and houses the cumulus covered oocyte. The follicle is made of several layers of cells and tissue; granulosa, membrane propria, theca interna (vascular), theca externa and the stroma.

1.1.4. Oestrous cycle, oocyte fertilisation, pregnancy, parturition and the desire to increase pig production

In the pig, the oestrous cycle lasts 21 days on average and begins at the onset of puberty between 150–220 days. This includes a two-day oestrus period in which the female is accepting of the male for mating. In agriculture, initial insemination usually begins at the third oestrous cycle following puberty (Soede *et al.*, 2011).

In the uterine horn, spermatozoa from the boar fertilise the ovulated oocyte, forming a zygote which subsequently cleaves and develops into an embryo during the preimplantation period. The average duration of gestation in the pig is 114–116 days, with conceptus attachment occurring around gestational day 18 leading to the formation of an epithelialchorionic placenta (Montiel *et al.*, 2013; Geisert *et al.*, 2015). Following pregnancy, depending on whether the breeding herd (in the UK) is housed indoors or outdoors, the pig can give birth to 12.4–14.2 piglets in a litter in total, of which 12.0–13.2 piglets are alive[1]. The sows go through a 16–40-day lactation period (depending on production system and country/legislation) before the piglets are weaned (Soede *et al.*, 2011). In the UK, again depending on whether the breeding herd is housed indoors or outdoors, a sow can wean 10.0–11.6 pigs in a litter[1]. Oestrus occurs again between 3–11 days after weaning.

Although pigs give birth to large litters, there is considerable reproductive loss which is represented by the high ovulation rates compared to the number of pigs weaned per litter. This is biologically inefficient and an economic loss to the agricultural industry. Therefore, this thesis focuses on understanding components of the ovary that are important to producing a good quality oocyte and subsequently a good quality embryo that is more likely to survive throughout pregnancy and the weaning period.

[1] June 2017 figures from Agriculture and Horticulture Development Board (AHDB) Pork website; https://pork.ahdb.org.uk/prices-stats/costings-herd-performance/

1.2. Oocyte Maturation and Folliculogenesis

1.2.1. Resumption of meiosis

During porcine embryonic development, 18 days postcoitum (PC), primordial germ cells migrate to an area (border of mesonephros) that will subsequently become the genital ridge/primitive gonad at 24–25 days PC (Black and Erickson, 1968). The primordial germ cells then proliferate and break down to form primordial follicles 68 days PC (Oxender *et al.*, 1979). These primordial follicles contain oogonia that begin first meiosis 40–50 days PC to form primary oocytes up until 100 days PC and by 15–20 days postpartum, 99% of these are arrested at the diplotene stage of prophase I of the first meiosis, characterised by a large nucleus known as the germinal vesicle (GV) (Black and Erickson, 1968; Oxender *et al.*, 1979).

During the oestrous cycle, the oocytes grow in size but remain at the GV stage. Fully grown oocytes remain arrested in the first prophase due to low activity of the maturation-promoting factor (a complex consisting of cyclin dependent kinase 1 and cyclin B) that regulates G2/M transition of the cell cycle (Rimon-Dahari *et al.*, 2016). It is only towards the end of an oestrous cycle that the oocytes resume meiosis. The resumption of meiosis is indicated by the germinal vesicle breakdown (GVBD) stage which signals that the oocytes have completed the first meiosis. Oocyte maturation then continues to the metaphase II (MII) stage, characterised by the production of a first polar body and where the oocytes have the developmental competence to be fertilised and are ovulated.

In addition to the nuclear maturation that occurs, the oocytes also undergo cytoplasmic maturation. This involves the metabolism of carbohydrates and lipids, mitochondrial relocation, reduction of oxygen radicals, the accumulation of follistatin, epigenetic programming, communications between cumulus cells and the oocyte, and the secretion of oocyte-derived growth factors (Krisher, 2013).

1.2.2. Folliculogenesis and hormonal regulation during the oestrous cycle

At birth, a female's ovary contains millions of primordial follicles (**Figure 1.2a**) which house the primary oocytes. These follicles consist of a thin basal lamina enclosing a thin layer of granulosa cells. Puberty occurs following a reduction in the activity of neural inhibitory mechanisms and/or a decrease in the negative feedback action of ovarian steroids which leads to the stimulation of pulsatile GnRH release, episodic LH secretion and ovarian activity (Kyriazakis and Whittemore, 2006). After puberty, circulating levels of follicle stimulating hormone (FSH) initiate the growth of a subset of these follicles by stimulating the proliferation of granulosa cells, their production of oestradiol and the expression of luteinising hormone (LH) receptors (Rimon-Dahari et al., 2016). During follicle growth the basal lamina increases, and the granulosa cells proliferate to form a multi-layered stratified epithelial-like structure surrounding the oocyte, forming the secondary follicle (**Figure 1.2**Error! Reference source not found.**b**). Under the influence of gonadotrophins, the granulosa cells release secretions which accumulate in the intercellular spaces, and this continued secretion results in the dissociation of granulosa cells, leading to the formation of the fluid filled antrum. The presence of the antrum marks the tertiary status of the follicle (**Figure 1.2c**). Continued proliferation of the granulosa cells as well as the appearance of the two layers of theca cells additionally mark the presence of the fully-grown follicle, referred to as a Graafian follicle or pre-ovulatory follicle (**Figure 1.2d**). A pre-ovulatory follicle can be over 5 mm in length and the proportion of these follicles increases from day 15 to day 20 of the oestrous cycle in a cyclic pig (Ryan et al., 1994). Additionally, the size of the follicle can often be an indicator of subsequent oocyte developmental competence, with larger follicles from pre-pubertal gilts containing oocytes that are more likely to develop into blastocysts following parthenogenetic activation (PA) (Bagg et al., 2007). Ovulation then occurs which involves the rupture of the follicle, thereby releasing the oocyte into the oviduct (**Figure 1.2e**).

The granulosa cells produce oestrogens by a process known as steroidogenesis, in which enzymes (CYP11A1, 3β-HSD, CYP17 and CYP19), under stimulation of FSH, aromatise androgens into oestrogens (mainly oestradiol and oestrone). The androgens

(mainly testosterone and androstenedione) were produced from acetate and cholesterol by the theca cells (stimulated by LH). Some of these androgens can also be aromatised into oestrogen in the theca cells. As part of the aromatisation process, the hormone progesterone is also produced (Rimon-Dahari et al., 2016).

The growth of the follicles is initiated by the presence of circulating FSH. As the follicles grow, granulosa cells produce oestrogens such as oestradiol which are FSH suppressors. The increase in oestradiol produces a positive feedback effect on the hypothalamic-pituitary-gonadal (HPG) axis (**Figure 1.3**), by increasing the release of gonadotrophin-releasing hormone (GnRH). This leads to an increase in the levels of oestradiol around day 16–18 as well as the sharp increase in levels of LH around day 18–20, also known as the LH surge. The peak of oestradiol causes a negative feedback effect on the HPG axis, which suppresses the release of GnRH and therefore reduces the levels of FSH. The LH surge incites the resumption of meiosis in the oocyte and the expansion of the cumulus (Rimon-Dahari et al., 2016). Additionally, it is the LH surge that results in the rupture of the follicle at ovulation, thereby expelling the COC into the oviduct. Oestradiol production decreases as ovulation occurs, removing the negative feedback on the HPG axis. Therefore, FSH concentration increases 1–2 days after ovulation which induces a wave of synchronised follicle development and another oestrous cycle.

Following ovulation, the residual granulosa cells and thecal cells undergo terminal differentiation to create the corpus luteum in a process known as luteinisation. This process is accompanied by the infiltration of the thecal blood vessels into the inner part of the follicle and the breakdown of the basal lamina (Rimon-Dahari et al., 2016). The corpus luteum secretes progesterone, which is involved in the maintenance of pregnancy in the uterus and exerts a positive feedback effect on the HPG axis. The concentration of progesterone peaks 8–10 days after ovulation, which eventually provides a negative feedback effect that suppresses the secretion of the gonadotrophins (Soede et al., 2011). Regression of the corpus luteum occurs 15–16 days after ovulation to develop a corpus albican (Ziecik et al., 2017).

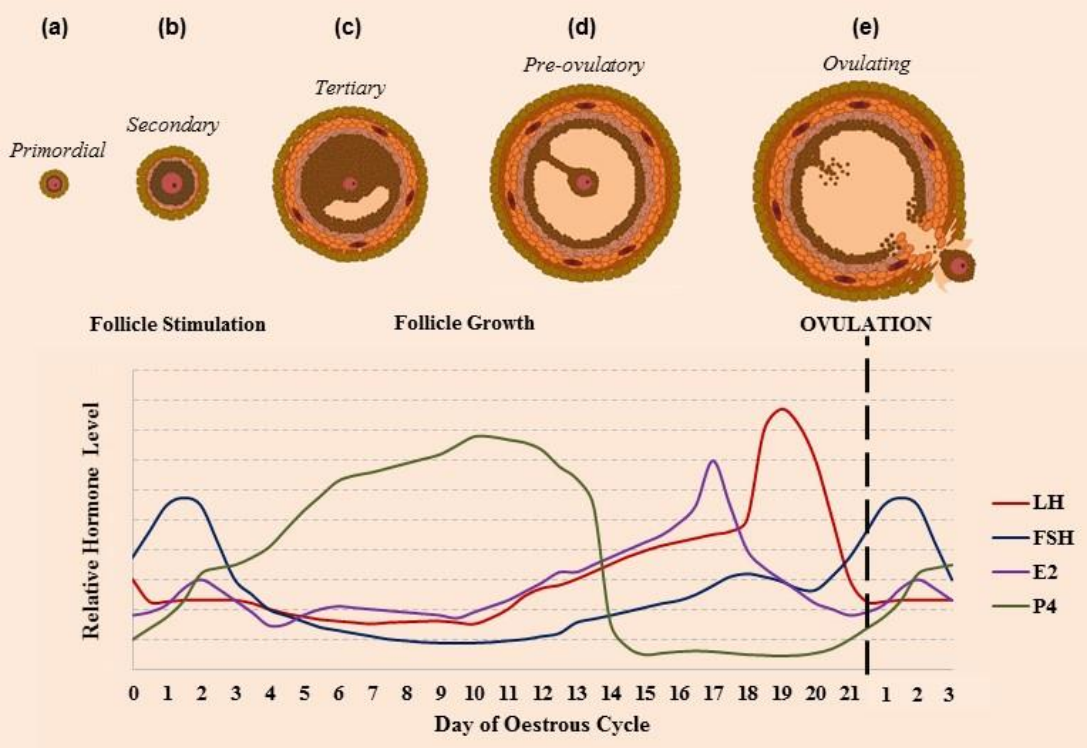


Figure 1.2. Schematic of hormone regulated folliculogenesis.

The stages of folliculogenesis from initiation of the primordial follicle (a) to a secondary follicle (b) induced by the increase in follicle stimulating hormone (FSH, blue). The secondary follicle expands to the tertiary (antral) follicle (c) and then eventually to the pre-ovulatory follicle (d). This is accompanied by the increase in progesterone (P4, green) which is involved in preparing the endometrial lining. Oestradiol (E2, purple) levels also increase which initiates the rapid release of luteinising hormone (LH, red). This LH surge results in the rupture of the follicle, an event known as ovulation (e). The relative hormone levels show the within hormone changes during the cycle and are not necessarily relative to each other; E2 concentrations are in pg/mL whilst P4, FSH and LH are in ng/mL. Adapted from Jarrett and Ashworth, (2018).

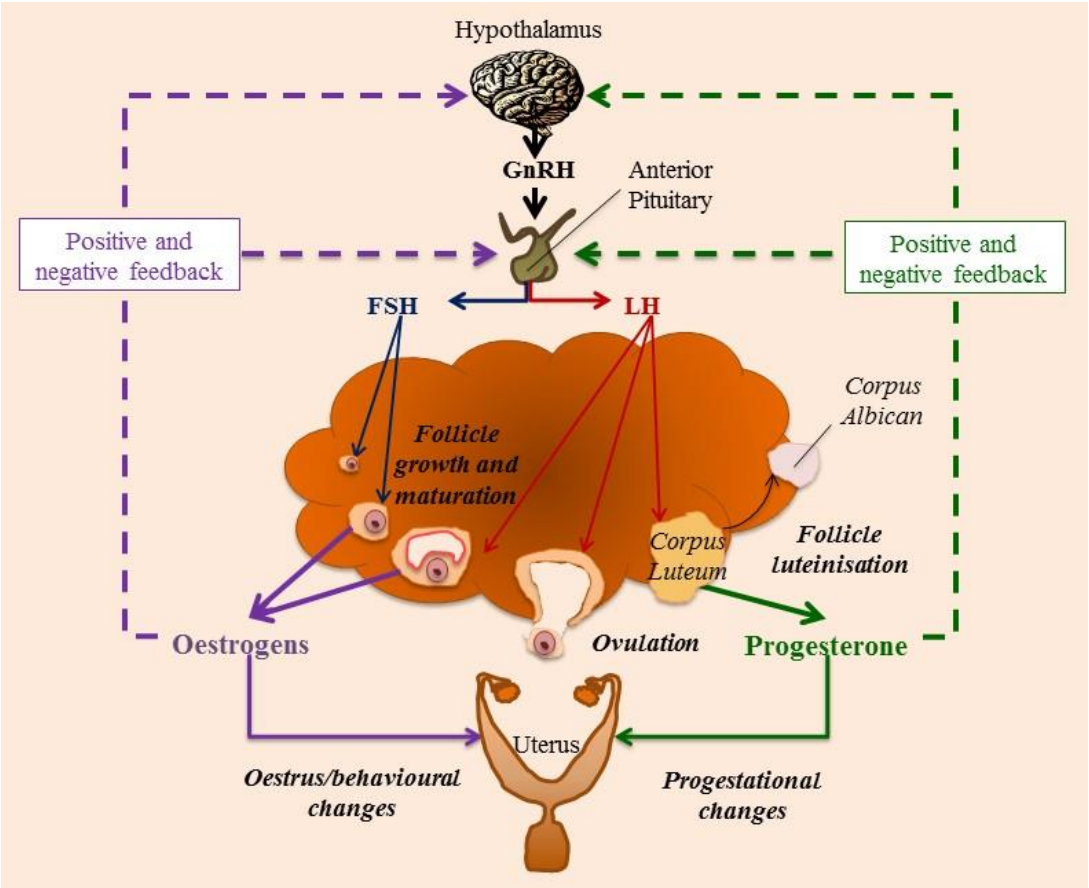


Figure 1.3. Schematic diagram showing the hypothalamic pituitary action on folliculogenesis.

In the hypothalamus, gonadotrophin releasing hormone (GnRH) is produced and is transported to the anterior lobe of the pituitary gland. There it stimulates gonadotroph cells to secrete follicle stimulating hormone (FSH, blue) and luteinising hormone (LH, red). FSH stimulates the development of follicles, and as the follicles develop, they release oestradiol/oestrogens (purple), which initiates a positive feedback on the hypothalamus and anterior pituitary gland. This results in an increase in GnRH pulses and therefore oestradiol and LH levels. This surge of LH induces ovulation. The accumulation of oestradiol exerts a negative feedback effect on the hypothalamus, reducing the secretion of GnRH and subsequently FSH and LH, and decreases the production of oestrogens. The *corpora lutea* secrete progesterone (green) which initially exerts a positive feedback effect on the hypothalamic-pituitary-gonadal (HPG) axis before inducing a negative feedback on the HPG axis and decreases the frequency of GnRH pulses.

1.2.3. Bidirectional inter-cell communication within the ovarian follicle

Within the ovarian follicle, a series of bidirectional communications occur between the oocyte and the surrounding somatic cells to regulate the earlier stages of folliculogenesis and oocyte maturation before these processes become increasingly gonadotropin-dependent. These communications are regarded as bidirectional as the growing follicles support oocyte maturation and progession of meiosis whilst the oocyte itself influences follicular growth including granulosa cell proliferation and differentiation and the production of the extracellular matrix and steroid hormones (Kidder and Vanderhyden, 2010).

Many of the molecules involved in these communications are cytokines, particularly members of the transforming growth factor (TGF) superfamily. These are polypeptide growth factors that bind to receptor complexes to initiate a signalling cascade involving Smad proteins, thereby activating Smad target genes (Wrana and Attisano, 2000; Rimon-Dahari *et al.*, 2016). The main TGFs involved in follicle development and that have been described in the pig and include anti-Müllerian hormone (AMH), bone morphogenic proteins (BMP)-4 and BMP-15, activins, TGF-β and growth differentiation factor 9 (GDF9) (Engelhardt *et al.*, 1992; Singh *et al.*, 1993; van den Hurk and van de Pavert, 2001; McCoard *et al.*, 2002; Brankin *et al.*, 2005; Zhu *et al.*, 2008; Sriperumbudur *et al.*, 2010). However, it is mainly rodent studies that have indicated the functions of these molecules.

Inhibition of follicular growth

The main function of AMH (produced by the granulosa cells of pre-antral and small antral follicles) is to suppress the recruitment of primordial follicles and the development of early follicle development to the pre-antral stage (Dunlop and Anderson, 2014). The specific mechanism by which AMH does this is uncertain, though rat studies suggest that FSH sensisitivy is suppressed and TGF-β signaling pathways are downregulated by AMH (Nilsson *et al.*, 2007; Dunlop and Anderson, 2014). Additionally, recent evidence from mouse studies showed that AMH induced

the granulosa cell expression of microRNAs, *miR-181a* and *miR-181b*, which target FSH signalling and TGF-β factors that promote follicular growth (Hayes *et al.*, 2016).

Initiation and progression of oocyte growth

KIT ligand (KL), is expressed in the granulosa cells of primordial follicles of pigs (Brankin *et al.*, 2004; Moniruzzaman and Miyano, 2007), mice and humans (Laitinen *et al.*, 1995; Ismail *et al.*, 1996). Its receptor is expressed in primordial oocytes of neonatal (10–20 days) and prepubertal (6 months old) pigs (Moniruzzaman and Miyano, 2007) and in oocytes of all stages of follicular development in mice and human ovaries (Horie *et al.*, 1991; Motro and Bernstein, 1993). Inhibition of this receptor suppressed FSH-induced growth of mouse oocytes, indicating the importance of this ligand's activity in the oocyte (Thomas *et al.*, 2005).

KL has been shown to activate the phosphatidylinositide 3-kinase pathway in the mouse oocyte, leading to the recruitment of primordial follicles (John *et al.*, 2009). When this pathway is activated in pig oocytes, increased phosphorylation of the serine/threonine-specific protein kinase protein kinase B occurs, which results in increased cell survival and proliferation as well as the inhibition of apoptosis (Prochazka *et al.*, 2012)(Dunlop and Anderson, 2014). The pathway is further stimulated by oocyte-derived GDF9 and BMP-15, which work synergistically to regulate KL expression in granulosa cells (Wang *et al.*, 2012, 2013a; Dunlop and Anderson, 2014).

Initiation and progression of follicular growth

BMP-4 and leukaemia inhibitory factor (LIF) and fibroblast growth factor (FGF) stimulate the transition of primordial follicles to primary follicles (Dang-Nguyen *et al.*, 2014; Dunlop and Anderson, 2014). BMP-4, derived from surrounding stromal-interstitial cells, acts on the oocyte and/or granulosa cells of primordial follicles to promote cell survival in developing follicles of rat ovaries (Nilsson and Skinner, 2003). Granulosa-derived LIF and oocyte-derived FGF enhances the expression of KL in granulosa cells of rats; KL (along with with FGF) then stimulates the primordial to

primary follicle transition by promoting the development of the oocyte and the proliferation of the granulosa cells and the stromal cells (Nilsson *et al.*, 2002; Nilsson and Skinner, 2004).

Following activation of primordial follicles, the primary follicles develop into pre-antral follicles, which require further proliferation of the granilosa cells. Activins secreted by granulosa cells promote the expression of FSH receptor on these cells, enhancing their proliferation (Knight *et al.*, 2012). Conversely, inhibins are produced by granulosa cells of antral and pre-ovulatory follicles and and suppress FSH secretion, as well as downregulate activin and BMP signalling events (Knight *et al.*, 2012). Therefore, towards late follicle growth, the follicles become increasingly dependent on circulating gonadotropins for their development prior to ovulation.

1.3. Follicular Fluid: Microenvironment for the Maturing Oocyte

1.3.1. Formation of follicular fluid from osmotic pressure

During folliculogenesis, granulosa cells produce secretions that eventually accumulate in areas with few cell-cell contacts, thereby separating a selection of the granulosa cells. This is the beginning of the follicular antrum, which initially expands through apoptosis of the granulosa cells. The granulosa cell secretions include hydrophilic and highly negatively charged molecules such as hyaluronan, versican and proteoglycans, chondroitin sulphate, dermatan sulphate and glycosaminoglycans, which increase the osmotic pressure within the antrum (Familiari *et al.*, 1986; Rodgers *et al.*, 1995, 2003; Clarke *et al.*, 2006). However, it is not only secretions from the granulosa cells that form the follicular antrum. During follicle development, follicles acquire two-capillary vascular networks located in the theca interna and externa layers, which eventually form multi-layered networks, that never penetrate to the granulosa cell layers due to the basement membrane that is located between them and the theca cells (Rimon-Dahari *et al.*, 2016). The osmotic gradient between the follicle and the vascular network surrounding it results in the transport of fluid (containing molecules less than 100 kDa) through the blood-follicle barrier (Rodgers and Irving-Rodgers, 2010). Aquaporins (1, 5 and 9 in pigs) within the granulosa cells also enable the transport of water and larger serum proteins into the antrum (Skowronski *et al.*, 2009).

1.3.2. Follicular fluid composition and the functions of follicular fluid components

As a product of secretions from granulosa cells, theca cells and the oocyte as well as being a plasma transudate, follicular fluid (FF) is a complex mixture of several different molecules. These molecules have several functions in ensuring the adequate development of the oocyte and ascribe FF its role as the microenvironment for the oocyte.

Proteins and amino acids

One of the major components of porcine follicular fluid (pFF) are proteins, with average total concentrations ranging between 570–740 µg/mL depending on the size

of the follicle (Wu *et al.*, 2002). The composition of pFF is dynamic in nature and amongst the complex protein milieu are the cytokines and growth factors involved in inter-cell follicular communications (described in section 1.2.3 of this chapter) including epidermal growth factor (EGF), inhibin and activin (Genlin *et al.*, 1998). Other cytokines include insulin-like growth factor-1, which is involved in promoting the FSH-induced differentiation, proliferation and oestradiol and progesterone output of granulosa cells and LH-induced androgen synthesis by theca cells (Hammond *et al.*, 1988; Gregoraszczuk *et al.*, 2004). As part of (or in addition to) the intercellular communications, these growth factors are associated with an increase in cumulus expansion and maturation to MII following *in vitro* maturation (IVM) (Ding and Foxcroft, 1994; Illera *et al.*, 1998; Procházka *et al.*, 2000; Prochazka *et al.*, 2003).

Many proteins accumulate in FF of various species to prepare for the inflammatory response required for the rupture event at ovulation (Boots and Jungheim, 2015). The LH surge induces the production of prostaglandins, which stimulates the release of proteolytic enzymes to promote angiogenesis, hyperaemia and collagenases, and to degrade the follicle's connective tissue (Boots and Jungheim, 2015). One inflammatory peptide involved in vasodilation is bradykinin, produced by plasmaderived protease kallikrein in porcine ovaries (Kihara *et al.*, 2000; Kimura *et al.*, 2000). Another is C-reactive protein, which is a well-known marker for inflammation and its levels have been shown to be higher in human follicular fluid (hFF) of women undergoing controlled ovarian hyperstimulation (COH) (Orvieto *et al.*, 2004; Wunder *et al.*, 2005). Other common acute phase proteins present in FF are interleukins, particularly interleukin-6 and interleukin-10 in pFF which are involved in granulosa cell apoptosis (Maeda *et al.*, 2007a, b; Jatesada *et al.*, 2013). In FF of various species including human, horse and pig, the most abundant proteins are the inflammatory/acute phase proteins serum albumin, serum transferrin, haptaglobin, apolipoprotein A1 and immunoglobulins, which is similar to that of the abundant proteins found in plasma and serum (Fahiminiya *et al.*, 2011a; Ducolomb *et al.*, 2013). These acute phase proteins can also directly improve oocyte development *in vitro*. Supplementation of IVM medium with pFF fractions containing immunoglobulin and transferrin increased the proportion of MII oocytes after IVM and fertilization rate

during *in vitro* fertilisation (IVF), whilst supplementation with fractions containing serum albumin promoted GVBD and maturation to MII as well (Ducolomb *et al.*, 2013).

The component monomers of proteins are amino acids, and pFF is rich in many amino acids, with glycine, alanine and glutamine being among the most abundant in porcine follicles of various sizes (Chang *et al.*, 1976; Hong and Lee, 2007). Amino acids have various functions that include maintaining pH, osmosis and osmotic pressure and providing energy (Edwards *et al.*, 1998; Palacín *et al.*, 1998; Rose-Hellekant *et al.*, 1998). Therefore, supplementation of defined IVM medium with essential amino acid medium and non-essential amino acid medium was associated with an increase in monospermic fertilisation and male pronuclear formation and improved blastocyst development after IVF (Ka *et al.*, 1997; Hong *et al.*, 2004). Additionally, when added individually to the defined maturation medium, glutamine, aspartate and valine improved fertilisation following IVF, whilst asparate and asparagine stimulated male pronuclear formation, and the addition of arganine and alanine to IVM medium improved blastocyst formation (Hong and Lee, 2007).

Lipids and fatty acids

Lipids have several physiological functions including energy storage, signal modulation and formation of plasma and organelle membranes. Sub-classes of lipids found in hFF include phospholipids, sphingolipids, triglycerides and steroid lipids such as cholesterol (Bokal *et al.*, 2006; Cordeiro *et al.*, 2015). Lipids are regarded as being either hydrophobic or amphipathic, and it is their component fatty acids that provide these molecules with this feature.

Early studies identified the most abundant fatty acids in both FF and oocytes of several domestic species including porcine, bovine and ovine (McEvoy *et al.*, 2000; Sturmey *et al.*, 2009; Dunning *et al.*, 2010, 2014). Free fatty acids are circulated through the body either by binding to serum albumin, which acts as a carrier protein, or they are circulated in the form of carboxylic acid derivatives and triacylglycerol/triglyceride molecules, which are carried by high density lipoproteins. Therefore, fatty acids enter

the follicular antrum into FF through plasma-derived diffusion, though the exact mechanism is not yet known matrix (Dunning *et al.*, 2014). Intracellular triacylglycerol is then stored in cumulus cells and oocytes within lipid droplets surrounded by coat proteins such as Perilipin2, and the lipid droplet proteins facilitate lipase-mediated hydrolysis of triacylglycerol and release of fatty acids matrix (Dunning *et al.*, 2014).

In the ovary, fatty acids are metabolised by the process of β-oxidation in the mitochondria of oocytes and cumulus cells to produce adenosine triphosphate (ATP) for energy to be used in oocyte maturation, zygote cleavage and early embryo development (Sturmey *et al.*, 2009; Dunning *et al.*, 2010, 2014; Sanchez-Lazo *et al.*, 2014). The LH surge induces β-oxidation and begins with the activation of fatty acids into fatty acyl-CoA, catalysed by carnitine palmitoyl transferase I, attaching a carnitine molecule that enables the fatty acid to enter the mitochondrial matrix (Dunning *et al.*, 2014). There, carnitine is removed by carnitine palmitoyl transferase II and enters the β-oxidation spiral to produce multiple acetylcoenzyme A molecules, from which ATP is generated via the tricarboxylic acid cycle and the electron transport chain (Dunning *et al.*, 2010). During ovulation, energy use increases in maturing oocytes via the mitochondrial oxidation of free fatty acids as a more efficient use of ATP than glycolysis (Paczkowski *et al.*, 2013; Valsangkar and Downs, 2013; Dumesic *et al.*, 2014; Dunning *et al.*, 2014).

In addition to being a source of energy in the ovary, fatty acids (specifically linoleic acid, linolenic acid and arachidonic acid) are involved in the synthesis of hormone-like lipids known as prostaglandins, most notably prostaglandins E2 and F2-α in pigs which increase in abundance towards ovulation (Ainsworth *et al.*, 1975; Tsang *et al.*, 1979; Hunter and Poyser, 1985). This process involves the cyclooxygenase pathway which requires the consumption of two oxygen molecules and two reduced glutathione molecules, and the enzyme action of the prostaglandin synthase complex. In this reaction, cyclooxygenases (or prostaglandin endoperoxide synthase) convert arachidonic acid into the cyclic endoperoxide-containing prostaglandin G_2, which is then cleaved of its peroxide functional group to produce prostaglandin H_2 (Mollace *et al.*, 2005). These are unstable intermediate products, which are subsequently converted

into prostanoids, such as prostaglandins E2 and F2-α, by isomerase enzymes (Mollace *et al.*, 2005).

Despite the important functions that fatty acids have in the ovarian follicle, studies have illustrated the negative impacts of an accumulation of free fatty acids in FF. The hFF concentration of polyunsaturated fatty acids positively correlated with an oxidative stress biomarker and negatively correlated with *in vitro* embryo cleavage rate and mean blastomere number (Kazemi *et al.*, 2013). Supplementation of saturated fatty acids during oocyte culture had negative effects on the maturation, fertilisation and cleavage of bovine oocytes and on subsequent blastocyst yield (Leroy *et al.*, 2005). Elevated levels of free fatty acids in FF was associated with reduced proliferation of granulosa cells, delayed maturation and decreased fertilisation, cleavage and embryonic development in dairy cows along with an increased induction of endoplasmic reticulum stress markers and impaired nuclear maturation in mouse COCs (Jorritsma *et al.*, 2004; Yang *et al.*, 2012). However, these results are equivocal as Sinclair, (2008) and Aardema *et al.*, (2013, 2015) did not observe any differences in oocyte maturation, cleavage rates or blastocyst rates due to increased free fatty acids in bovine FF whilst Zachut *et al.*, (2010) observed higher cleavage rates in bovine oocytes matured in FF with increased ω3-fatty acids. It is unclear whether these differences are due to experimental differences or perhaps they are dependent on the relative composition of endogenous fatty acids in the FF.

Hormones

The importance of hormones and growth factors such as FSH, LH, EGF, oestradiol and progesterone in regulating the oestrous cycle and folliculogenesis have been discussed in section 1.2.2. Additionally, some of these hormones have been identified in pFF and their concentrations can be an indicator for sexual maturity with progesterone and androstenedione concentrations being higher in pFF of adult pigs compared to pFF of pre-pubertal pigs (Biggs *et al.*, 1993; Grupen *et al.*, 2003). As with proteins and fatty acids, there is also evidence that these hormones play direct roles in the maturation of oocytes. Large follicles of abattoir-derived ovaries that were associated with more MII oocytes following IVM and had higher pFF oestradiol and

progesterone concentrations compared to pFF from small follicles associated with fewer MII oocytes (Liu *et al.*, 2002).

Carbohydrates and polysaccharides

Glucose is a common sugar present in pFF and its concentration increases with follicle size (Chang *et al.*, 1976; Bertoldo *et al.*, 2013). The two main mechanisms involved in glucose metabolism are the pentose phosphate pathway (PPP) and glycolysis. The PPP produces nicotinamide adenine dinucleotide phosphate (NAHPH) for the reduction of the antioxidant glutathione as well as phosphoribosylpyrophosphate, which is a substrate for *de novo* purine synthesis important for oocyte meiosis (Sutton-McDowall *et al.*, 2010). Glycolysis of glucose produces another important carbohydrate, pyruvate, which can be used to produce energy in the form of ATP through the tricarboxylic acid cycle and oxidative phosphorylation (Sturmey and Leese, 2003; Sutton-McDowall *et al.*, 2010). Therefore, supplementation of IVM medium with inhibitors of these pathways was associated with decreased glutathione, NADPH, intra-oocyte ATP and MII oocytes, as well as increased cell apoptosis and reactive oxygen species (ROS) levels (Herrick *et al.*, 2006; Yuan *et al.*, 2016).

Subsequently, IVM systems supplemented with PPP and glycolysis inhibitors were associated with decreases in cleavage rate, blastocyst rate and blastocyst cell number following IVF (Herrick *et al.*, 2006; Yuan *et al.*, 2016).

Another mechanism involved in glucose metabolism is the polyol pathway which produces fructose and sorbitol as additional sources of energy (Sutton-McDowall *et al.*, 2010). The final known mechanism is the hexosamine biosynthetic pathway which produces the polysaccharide hyaluronan, a major component of the extracellular matrix of cumulus cells and is important for porcine COC expansion and subsequent maturation of porcine oocytes (Yokoo *et al.*, 2008; Nagyova, 2012).

Reactive oxygen species and antioxidants

ROS are oxygen-based metabolites present in FF at low levels, most likely as a byproduct of steroidogenesis, and are important messengers in modulating the

expression of genes involved in oocyte maturation (Basini *et al.*, 2008). However, at higher levels these molecules can contribute to oxidative stress which can lead to meiotic arrest, oocyte degeneration and oocyte apoptosis (Tatemoto *et al.*, 2000). Therefore, ROS can be regulated by antioxidants which are also present in FF. Examples of ROS found in pFF include superoxide anions, hydroxyl radicals and hydrogen peroxide whilst antioxidants include superoxide dismutase and glutathione (Tatemoto *et al.*, 2000; Basini *et al.*, 2008).

Summary: a dynamic and complex environment

The composition of pFF and FF of other mammals is multifaceted and is in continual flux depending on the stage of folliculogenesis and the health of the individual. Several different types of molecules compose the ovarian envrionement of the oocyte and the composition influences the success of that oocyte (**Figure 1.4**). Studies have shown that it is not simply the abundance of one type of molecule and the lack of another that leads to good fertility, but that it is a balance of these various molecules. With this in mind, it is understandable that attempts to mimic the ovarian follicular environment are still being carried out with varying success.

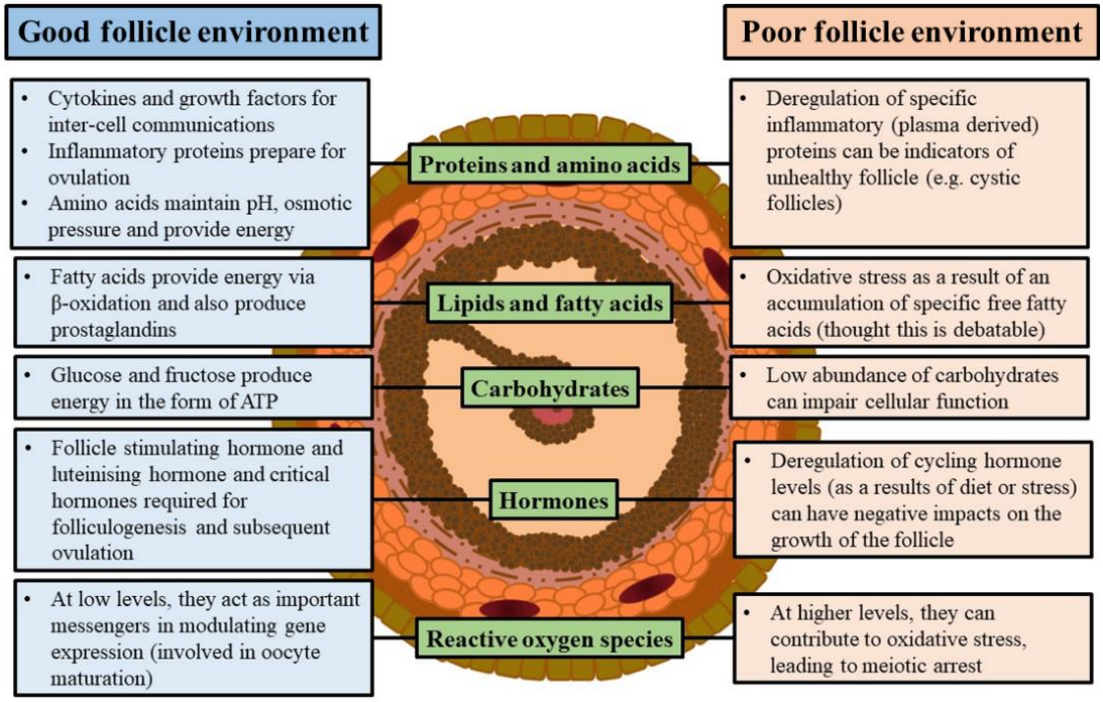


Figure 1.4. Summary diagram of how the different main components of follicular fluid influence the intra-ovarian environment.

Follicular fluid is composed of several different types of molecules including proteins and amino acids, lipids and fatty acids, carbohydrates, homrones and reactive oxygen species. The quality of the follicle depends largely on the relative amounts of these different molecules.

1.3.3. Recreating *in vivo* conditions for *in vitro* maturation of oocytes

The IVM of oocytes is the first of three stages of the *in vitro* production of embryos, which is followed by the IVF of mature oocytes and the *in vitro* culture of subsequent embryos. Prior to IVM, oocytes are collected before ovulation, making them developmentally immature. Therefore, IVM functions to mimic the later stages of the oocyte maturation process that occurs in the ovarian follicle post LH surge. As with *in vivo* maturation, IVM of oocytes is critical for the success of the embryo. Therefore, the particulars of how the technique is carried out are critical for the cellular processes that occur during maturation and can determine overall reproductive success.

The technique was developed for pig oocytes in the mid-1960s (along with IVM of mouse, sheep, cow, rhesus monkey and human oocytes), and was steadily optimised in the years that followed (Edwards, 1965). It was only in the late 20th century that porcine oocytes matured by IVM were successfully fertilised by IVF, producing embryos that reached the blastocyst stage, which was made possible by increasing the temperature of the incubators to pig body temperature (Mattioli *et al.*, 1989; Yoshida *et al.*, 1990).

Part of refining the *in vitro* environment was to analyse the effects of supplementation of medium with elements of the ovarian follicle, particularly the FF. An early study identified no detrimental effects in the proportion of MII porcine oocytes cultured in 100% pFF compared with culture with Tissue Culture Medium 199 (Racowsky and McGaughey, 1982). Following the ability to fertilise oocytes matured *in vitro*, the inclusion of pFF in IVM medium not only improved nuclear and cytoplasmic maturation of the oocytes but also increased normal fertilisation and cleavage rates following IVF (Yoshida *et al.*, 1992). In particular, pFF supplementation during the first half of a 44-hour IVM protocol stimulated cumulus expansion whilst pFF supplementation during the second half increased progesterone production and the proportion of MII oocytes (Grupen and Armstrong, 2010).

The inclusion of different components in FF into IVM systems can also improve IVM and IVF outcomes. The results of pig IVM studies have shown that the inclusion of

EGF, LH and FSH and EGF together increased the percentage of MII oocytes and improved fertilisation and male pronuclear formation (Singh *et al.*, 1993; Ding and Foxcroft, 1994; Illera *et al.*, 1998). Additionally, supplementation with the follicle shell along with hormones alone increased the rates of meiotic maturation to MII as well as cleavage rates following PA (Lui *et al.*, 1997). Subsequently, the results of these studies along with studies that supplemented medium with other known important factors associated with pFF (see section 1.3.2.), such as amino acids, led to the optimisation of general IVM protocols to include these factors as common supplements to oocyte IVM medium (Sutton *et al.*, 2003; Krisher, 2013). This also includes the addition of glucose and pyruvate which was associated with improved nuclear and cytoplasmic maturation of porcine oocytes and increased glutathione production (Sato *et al.*, 2007; Funahashi *et al.*, 2008). In an attempt to mimic *in vivo* conditions further, (Kawashima *et al.*, 2008) designed a culture system for porcine COCs in which hormones were added sequentially (FSH, oestradiol, progesterone and cultured in fresh medium with LH, EGF and progesterone) which resulted in a greater proportion of COCs with full expansion, fewer apoptotic cumulus cells and improved developmental competence to the blastocyst stage following IVF.

Another hormone that has gained significant interest is melatonin, which is also present in pFF, and has been shown to be present in lower levels in large follicles compared to small follicles (Shi *et al.*, 2009). Maturation medium supplemented with melatonin (ten times the concentration found in pFF) increased cleavage rates, blastocyst rates and blastocyst cell numbers (Shi *et al.*, 2009). Additionally, more recent studies have observed the beneficial effects of melatonin supplementation into embryo culture medium (Do *et al.*, 2015; Li *et al.*, 2015; Nakano *et al.*, 2017).

Despite the attempts to mimic the maturation conditions of the ovarian follicle, the rates of pronuclear formation and blastocyst formation in IVM oocytes is still significantly lower than their *in vivo* counterparts whilst having increased incidence of polyspermic fertilisation (Krisher, 2013). Additionally, only approximately a third of oocytes develop to the blastocyst stage following IVM and these blastocysts often have suboptimal cell numbers, cryotolerance and post-transfer survival compared with *in*

vivo produced blastocysts (Krisher, 2013). Therefore, there is still room to improve the *in vitro* environment of a maturing oocyte. One way to find biomarkers for good quality oocytes is to assess the *in vivo* conditions that produce them.

1.4. Assessing the composition of Follicular Fluid

1.4.1. Analyses of specific follicular fluid components

There have been several techniques that have been utilised to measure the various components of pFF. Many of the earliest studies that have assessed molecules in pFF (and FF of other domestic species) utilised radioimmunoassay to measure hormones such as cortisol, relaxin, oestradiol, progesterone and testosterone (Cook *et al.*, 1977; Mahajan and Little, 1978; Ainsworth *et al.*, 1980; Mahajan *et al.*, 1980; Matsumoto and Chamley, 1980; Fleming and McGaughey, 1982; González-Serrano *et al.*, 2015), polyacrylamide gel electrophoresis to measure proteins (Cook *et al.*, 1977; Mahajan and Little, 1978; Mahajan *et al.*, 1980), gas-chromatography to measure cholesterol activity (Yao *et al.*, 1978), and fluorescence based assays to determine concentration of proteoglycans and oocyte maturation inhibitor (Stone *et al.*, 1978; Yanagishita *et al.*, 1978). These early studies established the presence of these molecules in pFF as well as some of the chemical and physical properties of pFF such as pH (Knudsen *et al.*, 1978). Additionally, they illustrated the stimulation of gonadotrophins on pFF steroid hormone concentrations (Ainsworth *et al.*, 1980), identified potential proteins that inhibit gonadotrophic stimulation (Kling *et al.*, 1984), and observed changes in steroid, inhibin and oocyte maturation inhibitor concentrations during the oestrous cycle (Wiel *et al.*, 1983; Xie *et al.*, 1990). Many of these techniques are still used in contemporary studies and whilst these techniques are reliable, they are limited in providing a detailed description of the dynamic composition of FF. To fully describe how a treatment or a disorder alters the microenvironment for a maturing oocyte, a more comprehensive strategy should be employed.

1.4.2. Mass spectrometry and ‘omics’ analyses

Omic analyses involve the characterisation and quantification of different molecules in specific cells, tissues or fluids to infer underlying biological processes. There are different types of omic analyses including proteomics, metabolomics, transcriptomics and genomics, which analyse a sample’s protein composition, metabolome, transcript activity and genome respectively. The majority of omics studies on pFF have been

proteomic and metabolomic analyses, whist transcriptomic and genomics have been carried out on oocytes (Toms *et al.*, 2014; Budna *et al.*, 2017a, b; Tao *et al.*, 2017). Due to its sensitivity, the mass spectrometer is an ideal tool to detect the presence of subtle changes in protein and metabolite compositions of the fluids and has become an attractive method for comparative studies. The information obtained from these analyses can provide indicators of metabolic perturbations or follicle health and development.

1.4.3. Proteomic analyses on human follicular fluid

Proteomic analysis in various aspects of medical and animal science has grown rapidly in the last 25 years, particularly in studies involving the detection of potential biomarkers. The study of proteins and their associations to particular treatments, characteristics or diseases is important as protein functions are central to biological processes and are therefore indicative of any fluctuations in those biological processes.

The majority of ovarian proteomic studies have been carried out on hFF, sourced from consenting IVF patients, with those receiving treatment for male factor infertility often providing the control samples. This is due to the expansion of reproductive medicine and the increasing aspiration for people to understand female fertility and reproductive disorders, as well as improving the modest success rates of human IVF. Proteomic profiling of hFF using mass spectrometry techniques is well established and has garnered considerable interest, with many of the earlier studies attempting to characterise the hFF proteome and identify novel proteins in the hFF of fertile IVF patients (Anahory *et al.*, 2002; Lee *et al.*, 2005; Sim and Lee, 2008).

Proteins identified in hFF have highlighted the processes involved in follicle development and ovulation. Sensitive tandem mass spectrometry techniques identified hFF proteins involved in hormone secretion, coagulation, inflammation, immune response, extracellular matrix function, lipid metabolism, cholesterol metabolism and oxidative stress (Angelucci *et al.*, 2006; Hanrieder *et al.*, 2008, 2009; Jarkovska *et al.*, 2010; Twigt *et al.*, 2012; Ambekar *et al.*, 2013; Bianchi *et al.*, 2013; Shen *et al.*, 2017). These results indicated the increased diffusion of plasma proteins over the blood-

follicle barrier during follicle maturation as well as the immune response to inflammation and the protection of follicles from toxic injury.

Due to the interests in understanding successful IVF protocols, studies were carried out to find FF proteins associated with IVF outcome. Estes *et al.*, (2009) analysed hFF of IVF patients (average age of 30.5), undergoing their first cycle of IVF, to identify biomarkers associated with IVF outcome. The study revealed eight proteins that were increased in hFF of women in the "live birth" group and three that were decreased out of a total of 321 proteins separated compared to hFF of women with no pregnancy (Estes *et al.*, 2009). Similarly, Kushnir *et al.*, (2012) identified four hFF proteins unique to women with a normal pregnancy, seven with miscarriage and two with no pregnancy out of a total of 55 proteins common to all three groups. Using Ingenuity Pathway Analysis (IPA) software, these proteins were found to be associated with steroidogenesis and immune response (Kushnir *et al.*, 2012). IPA also matched proteins that were upregulated in pregnancy positive hFF with the complement system, coagulation system and acute phase response signalling pathways (Severino *et al.*, 2013). The effect of maternal age on IVF (and reproductive) success has been well established, with decreased viability of oocytes and embryos, as well as failed implantation associated with increasing age (Alviggi *et al.*, 2009). These reproductive disadvantages have also been represented in hFF, with the down-regulation of inflammatory proteins in the hFF of older women (38–42 years old) compared to the hFF of younger women (20–32 years old) (Hashemitabar *et al.*, 2014).

Proteomics has also been used to identify proteins and pathways associated with reproductive disorders. Coagulation factors were identified as being lower in hFF of recurrent spontaneous abortion patients compared to normal hFF of normal patients, indicating their role in maintaining normal pregnancy (Kim *et al.*, 2006). Differentially expressed proteins involved in glucose and lipoprotein metabolism, cell proliferation and apoptosis and insulin resistance were identified between hFF of normal women and hFF of women with Polycystic Ovary Syndrome (PCOS) (Dai and Lu, 2012; Ambekar *et al.*, 2015). Enrichment in proteins involved in oxidative stress response and apoptosis was detected in hFF of IVF patients with endometriosis who became

pregnant compared to controls and to women with endometriosis who did not become pregnant (Lo Turco *et al.*, 2013). In a similar study, the main processes identified from hFF protein profiles of fertile IVF patients were vitamin transport, inflammation and immune response, whilst coagulation processes and sterol metabolism were the main processes in women with endometriosis (Regiani *et al.*, 2015).

Treatment with medication can also affect the hFF proteome. Differentially expressed proteins, including ceruloplasmin and complement C3, were detected between control women and women with ovarian hyperstimulation syndrome, a condition that develops usually as a result of injection with human chorionic gonadotrophin (hCG) (Jarkovska *et al.*, 2011). Similarly, complement components and coagulation factors were significantly different between hFF before hCG injection and hFF post-hCG as well as hFF of infertile women undergoing COH compared to infertile women with natural cycles (Wu *et al.*, 2015; Zamah *et al.*, 2015; Lim *et al.*, 2017). A difference in FF proteins was also observed between women with poor ovarian response following COH and FF of control women who also underwent COH (Oh *et al.*, 2017). Additionally, women undergoing folic acid supplementation had lower C-reactive protein and apolipoprotein E, but higher high density lipoprotein (HDL) apolipoproteins, indicating the suppression of inflammatory processes and upregulation of HDL pathways (Twigt *et al.*, 2015).

Therefore, it is clear that proteomic research bears great potential in reproductive studies. Additionally, mass spectrometry and bioinformatic techniques can been used to identify novel and known proteins associated with molecular pathways implicated in reproductive processes.

1.4.4. Proteomic analyses on follicular fluid of other species

Whilst the use of proteomic technologies in studying hFF for identifying therapeutic targets are relatively well-established, the same cannot be said for studying FF from other species. The advantages, milestones and limitations of proteomics in relation to animal research, particularly in pigs, have been previously discussed (Lippolis and Reinhardt, 2010; Soares *et al.*, 2012). There are universal factors that must be

considered when carrying out a proteomic analysis, regardless of whether the samples are from humans or animals. These include the limitation of mass spectrometer (as with any technique), the vast or small number of proteins in a sample and the dynamic range of a sample.

Although proteomic research in animal sciences is growing, there are more limitations associated with large animal samples than with human samples. One major hurdle is overcoming the dynamic range of biological fluids such as FF, plasma or serum and this proteome density is in part the result of post-translational modifications and alternate splicing of genes (Lippolis and Reinhardt, 2010). The composition of human serum, plasma or FF is also dynamic, but they are usually depleted of abundant proteins using human-specific immunodepletion kits, which are not as efficient when used with FF of other species. Another limitation of proteomics in animal research is the quality of genomic databases available. Unlike the Human Genome Project, the annotation of animal sequences is less complete and therefore the confidence in the protein databases for species such as the pig is lower. Thirdly, there are limitations in validation techniques, such as the availability of western blotting antibodies and enzyme linked immunosorbent assay kits, specific for non-rodent animals.

However, there are some advantages to carrying out studies with animal FF. As most hFF is obtained from donors at IVF clinics, it is difficult to obtain hFF from follicles from different sizes (at different developmental stages) and the hFF collected would have been collected from women undergoing artificial/stimulated cycles. However, studies on animal samples such as yak, goat, buffalo and bovine FF have identified differences in protein composition between FF from follicles at different stages of development, and between FF of healthy and cystic follicles (Maniwa *et al.*, 2005; Tao *et al.*, 2014; Fu *et al.*, 2016; Ferrazza *et al.*, 2017; Paula Junior *et al.*, 2017). Additionally, proteomic analyses have identified differences in protein compositions between canine FF before and after the LH surge as well as FF of pre-pubertal lambs and ewes (Fahiminiya *et al.*, 2010; Wu *et al.*, 2017). This illustrated the sensitivity of FF composition to the female's reproductive maturity and what stage of the oestrous cycle the follicle is in.

A couple of studies have also looked at how protein composition differed between pFF and porcine serum, and how composition differed between pFF from normal and abnormal follicles. Bijttebier *et al.*, (2009) identified upregulated proteins in pFF compared to porcine serum, which included antithrombin III, clusterin, fibrinogen β chain and inter-α-globulin inhibitor H1, indicating the flow of inflammatory proteins into the follicular antrum during folliculogenesis. Sun *et al.*, (2011) identified four downregulated proteins in pFF from small (2–4 mm) follicles compared to pFF from medium (>47 mm) follicles and eight proteins downregulated proteins in pFF from medium compared to pFF from large (>7–10 mm) follicles. This was indicative that changes in FF protein expression accompanies follicular maturation. There were also 20 different proteins between pFF from large follicles and cystic (≥21 mm) follicles; 11 of these proteins were downregulated in in pFF of cystic follicles and included plasma-derived proteins such as ceruloplasmin, albumin and haemoglobin (Sun *et al.*, 2011). Additionally, other plasma-derived proteins such as apolipoprotein A1, immunoglobulin and retinol binding protein were more abundant in cystic follicles, indicating a disruption in the regulation of the diffusion between the blood-follicle barrier (Sun *et al.*, 2011). Western blot confirmed retinol binding protein 4 levels as being higher in cystic follicles, making it a potential biomarker for porcine follicular cysts (Sun *et al.*, 2011). These studies provide encouragement that a proteomic analysis can be carried out on pFF and that the results can be used to ascertain physiological processes in the follicle.

1.4.5. Metabolomic analyses on human follicular fluid

Like proteomics, the metabolomic analyses of hFF is relatively well-established. However, unlike proteomics, due to the expansive nature of the metabolome, metabolomic analyses are usually more targeted in nature, by focussing on certain molecules such as steroid hormones, amino acids and other low molecular weight entities (Mcrae *et al.*, 2013).

The analysis of hFF metabolites, like proteins, aimed to identify the association of these molecules with reproductive/IVF success as well as reproductive disorders. Low molecular weight entities, such as glucose, lactate and pyruvate are often analysed

using nuclear magnetic resonance (NMR) spectroscopy (Piñero-Sagredo *et al.*, 2010). For example, NMR has identified the altered hFF levels of glucose, lactate, pyruvate, branched-chain amino acids and free fatty acids associated with different stages of follicle development, oocyte cleavage and pregnancy success (McRae *et al.*, 2012; Wallace *et al.*, 2012; O'Gorman *et al.*, 2013). However, mass spectrometry techniques have also been used to identify altered levels of free fatty acids, amino acids, dicarboxylic acids and cholesterol associated with COC morphology and IVF success (Jungheim *et al.*, 2011; Xia *et al.*, 2014; Mirabi *et al.*, 2017a).

Disorders such as PCOS and endometriosis are also common disorders that are assessed by both NMR and mass spectrometry-based techniques. An increase in levels of amino acids, androgens, sphingolipids, glycerophospholipids and free fatty acids as well as lower lactate and oestrogen was observed in hFF of obese PCOS patients compared to control PCOS patients (Naessen *et al.*, 2010; Niu *et al.*, 2014; Cordeiro *et al.*, 2015; Zhao *et al.*, 2015; Zhang *et al.*, 2017). Using this information, the authors were able to propose the potential up-regulation of glycolysis pathway, amino acid metabolism and fatty acid β-oxidation in ovaries of obese PCOS women (Zhao *et al.*, 2015; Zhang *et al.*, 2017). Additionally, an increase in lactate, insulin, phospholipid, sphingolipid, phosphatidylcholine levels and a decrease in glucose, lactate dehydrogenase, fatty acid and amino acid levels were detected in hFF of women with endometriosis compared to hFF of control women (Cordeiro *et al.*, 2015; Santonastaso *et al.*, 2017). This suggests the down-regulation of the glycolysis pathway, amino acid metabolism and fatty acid β-oxidation in ovaries of women with endometriosis.

There have also been studies that investigated how hFF fatty acid composition affects oocyte quality in the context of women's weight, though these have shown contradictory results as well. Valckx *et al.*, (2014) identified hFF free fatty acids that differed significantly between women of different Body Mass Index (BMI) groups whereas Mirabi *et al.*, (2017b) did not identify any differences in fatty acids associated with BMI.

1.4.6. Metabolomic analyses on follicular fluid of animals

The majority of non-human metabolomic studies have been carried out on bovine samples and have mainly targeted amino acid and fatty acid levels, known to be abundant in FF. This is likely due to amino acids and fatty acids being main energy reserves and the decrease in fertility of cows due to negative energy balance during the early weeks postpartum. These analyses have illustrated the association of metabolite composition with reproductive stage, reproductive capacity and feeding regimen. Different FF levels of fatty acids and amino acids have been identified between cows and heifers, between cows exposed to a short term fasting period and normal-fed cows, between cows in different days post-parturition and between lactating and nonlactating cows (Bender *et al.*, 2010; Aardema *et al.*, 2013; O'Doherty *et al.*, 2014; Forde *et al.*, 2016). In addition to these parameters, fatty acid concentrations have also been associated with blastocyst formation, oocyte competence and oestradiol and progesterone levels, in particular oestradiol:progesterone ratio, which denotes active and inactive follicles; a higher ratio being indicative of active follicles (Renaville *et al.*, 2010; Aardema *et al.*, 2013; Matoba *et al.*, 2014).

Whilst most of the metabolomic studies have been carried out on bovine FF, there have been a couple of studies carried out on pFF, measuring fatty acids, amino acids, carbohydrates and hormones. Pawlak *et al.*, (2012) identified the increased concentrations of stearic acid and palmitic acid in pFF of prepubertal gilts compared to cyclic pigs. Additionally, Bertoldo *et al.*, (2013) identified the increase in glucose and decrease in lactate, hypoxanthine and five amino acids in pFF of large follicles compared with pFF from small follicles as well as the correlation of these metabolites with pFF levels of progesterone, androstenedione and oestradiol.

Metabolomic studies have also been carried out on FF of other species. Wang *et al.*, (2013b) identified the different levels of cholesterol, cortisol, oestradiol, progesterone, amino acids and fatty acids in FF of rats fed soy isoflavones compared to control-fed rats. Additionally, Sessions-Bresnahan *et al.*, (2016) identified reduced levels of amines and amino acids, oxylipids, progesterone and hydroxyprogesterone and elevated levels of linoleic acid and stearic acid in obese mare FF compared with normal

mare FF. Differences in FF composition between the different species have also been observed. Gérard *et al.*, (2015) carried out a comprehensive study that characterized FF composition from the small and large follicles of pigs, cows and mares. Four and 14 metabolites differed in concentration between the FF from large follicles and FF of small follicles in cows and mares respectively, and both had higher levels of α- and βglucose in FF from large follicles (Gérard *et al.*, 2015). Additionally, pig FF was characterised by the presence of succinate and absence of citrate (Gérard *et al.*, 2015). Therefore, as with proteomics, metabolomics can be a useful tool is assessing the carbohydrate, amino acid and lipid profile of FF of various species.

1.4.7. Techniques for proteomics and metabolomics: mass spectrometry

The mass spectrometer is a useful tool in many proteomic and metabolomic experiements due to its sensitivity; it can work with samples as low as 10^{-12} g or 10^{-15} mol (Mittal, 2015). The technique works by first ionising the sample, accelerating the resultant ions, followed by ion spearation and then detection of those ions (Lössl *et al.*, 2016). This then generates a spectrum that acts as a "signature" for different molecules.

Ionisation occurs using an ion source such as electrospray ionisation (ESI) or matrix-assisted laser desorption/ionisation (MALDI), which provides the samples with a charge either through electron ejection, electron capture, protonation, cationisation, deprotonation or transferring a charge molecule from a condensed phase to the gas phase (Siuzdak, 2004). For example, ESI produces multiply charged analytes directly from a sample solution whilst MALDI uses a laser to ablate a matrix and analyte molecules from a metal plate into the mass spectrometer to produce singly charged ions (Lössl *et al.*, 2016). Resultant ions are then accelerated using an electromagnetic field so that they all have the same kinetic energy and are then are separted according to mass/charge ratio within the mass analyser using a magnetic field. The ions are then detected by an ion detector that converts the ion energy into electrical signals, which are transmitted to a computer (Siuzdak, 2004). Therefore, the mass spectrometer can measure the molecular and atomic masses of whole molecules as well as molecular fragments and atoms, and these masses correspond to known

molecular structures and atomic compositions, allowing for the identification of these molecules (Mittal, 2015).

Tandem mass spectrometry (MS/MS) is increasingly being used in biochemistry and molecular biology research. The technique uses a single instrument with two or more mass analysers, whereby the generated (precursor) ions are fragmented one or more times (by bombardment with an inert gas) into different charged and mass ions (product ions) in order to obtain highly specific detection readings (Mittal, 2015). However, when using MS/MS (and other mass spectrometry) techniques, there are factors that need to be considered depending on the tareget molecules being analysed.

Detecting proteins

Mass spectrometry and MS/MS has been heavily used in proteomics and is regarded as a well-established technique in the field. This includes the preparation techniques prior to detection. Generally, the protocol for preparing samples for proteomic analyses are quite similar (Chakraborty and Shekhar, 2017); (1) proteins are denatured, reduced and alkylated with agents such as urea, dithiothreitol and iodoacetoamide respectively; (2) proteins are then digested into peptides with a protease such as trypsin; (3) if quantifying, peptides are isotopically labelled and there are many commercially available labelling kits; (3) peptides are seprated by mass (and sometimes charge), usually through a chromatography technique, such as high-performance liquid chromatography.

Following detection of peptide masses, fragment ion spectra of the peptides are assigned to known protein sequences using platforms and software (such as Maxquant and Andromeda, respectively) to assign known protein sequences to the data (Chakraborty and Shekhar, 2017). Additionally, datatbases such as the Global Proteome Machine Database and Peptide Atlas, are used to make the data accessible and reusable (Perez-Riverol *et al.*, 2015). These detection platforms and database repositories often contain vast libraries of signature mass spectra of different proteins that are species specific. Some species have more comprehensive data, such as the

databases for the human proteome and rodent proteome, though the coverage for other species is expanding.

Detecting metabolites

Unlike proteomics, metabolomics describes a variety of molecule types that can be targeted, from lipids and fatty acids, to amino acids, to carbohydrates. This makes preparation of samples equally variable depending on which molecules are to be targeted (Gika and Theodoridis, 2011). Other factors to take into consideration include the small size of these molecules in comparison to proteins. Therefore, in many metabolomic studies that target fatty acid, amino acids and hormones, gas chromatography is used more than liquid chromatograpy as it is more sensitive (Fiehn, 2016).

Another factor to consider is the structural similarity of metabolites. For example, different fatty acids have similar number of carbon and hydrogen atoms, and usually only differ in the number and position of double bonds and/or hydrocarbon side chains. Therefore, a database of fragments with signature mass/charge spectra is not available for these molecules and they are instead identified *de novo* using known standards and quantified using internal standards (Gika and Theodoridis, 2011). Sunsequently, other spectrometry techniques have been utilised in metabolomic analyses, such nuclear magnetic resonance spectroscopy, magnetic resonance imaging and infrared spectroscopy (Mittal, 2015).

1.5. High Fibre Diet Improves Fertility in Female Pig

A practical means of improving female fertility can be to alter female pre-mating diet. Several studies have shown that a fibre diet is particularly effective in improving pig fertility since it is associated with a range of reproductive advantages, which can be observed macroscopically at the farm level, as well as the microscopic and molecular level. Depending on the geographical location the study was carried out, the dietary fibre source can be from konjac flour, oats, oat bran, soybean hulls, wheat bran, lupin or unmolassed sugar beet pulp, whilst the control diet can be largely based on barley, wheat or corn.

1.5.1. The most advantageous period of feeding the high fibre diet is during the oestrous cycle prior to mating

Oocyte maturation within the ovarian follicle is reliant on the supply of reproductive hormones and steroids, the concentrations of which depend on nutritional intake, making oocyte maturation sensitive to disruptions and perturbations in maternal health and wellbeing (Ashworth *et al.*, 2009; Krisher, 2013). Previous nutritional studies suggest that a diet enriched in fibre, fed prior to ovulation during the oestrous cycle, exerts a beneficial effect to pig fertility during the maturation of the oocyte in the ovary, which extends to the development of the blastocyst and subsequent production parameters such as litter size.

Oocytes from gilts fed a high fibre diet from days 1–19 of their third post-pubertal oestrous cycle had more MII oocytes following 46 hours of IVM culture compared to oocytes from control-fed gilts (Ferguson *et al.*, 2007). Additionally, pre-pubertal gilts fed a high fibre diet from three weeks before puberty stimulation until day 19 of their first oestrous cycle had nine times more MII oocytes than gilts fed the low fibre diet and five times more than the gilts fed the medium fibre diet (Weaver *et al.*, 2013). However, despite the alterations in oocyte quality, dietary fibre does not appear to affect ovulation rate (Glasgow *et al.*, 1999; Ferguson *et al.*, 2006, 2007; Renteria-Flores *et al.*, 2008; Weaver *et al.*, 2013).

The formation of a viable blastocyst is the ultimate indicator of a good quality oocyte and a blastocyst with more cells is considered to be better developed. Embryos flushed from gilts fed a high fibre-fed during their first oestrous cycle, and cultured in North Carolina State University Medium 23 for 6–7 days developed into blastocysts with more cells compared to embryos of control-fed pigs (Glasgow *et al.*, 1999). Additionally, more embryos were recovered from the high fibre-fed gilts compared to the control-fed gilts (Glasgow *et al.*, 1999). Following IVF, blastocysts produced from oocytes collected from high fibre-fed gilts during the first 19 days of their third oestrous cycle, had more cells than contemporary blastocysts produced from oocytes collected from control-fed pigs (Ashworth *et al.*, 2008).

The reproductive benefits of feeding the high fibre diet during the oestrous cycle were also confirmed with improvements to pregnancy outcomes. High fibre-fed-gilts and sows had increased embryo survival and a decreased incidence of intra-uterine growth restricted foetuses compared to gilts and sows fed either a control diet, a maintenance diet provided in three different amounts, or a diet with increased protein or starch prior to insemination (Ferguson *et al.*, 2003; Weaver *et al.*, 2013). High fibre-fed sows also showed later benefits, with more piglets born and more of them born alive, when the sows were fed the diet prior to ovulation from mid lactation (day 11) until breeding (day 25) and when the diet was fed to sows during lactation, litter growth rate and piglet body weight at weaning increased (Renaudeau *et al.*, 2003; Ferguson *et al.*, 2004).

The circulating levels of reproductive hormones, integral to reproductive function are also affected. High fibre-fed gilts had lower circulating oestradiol concentrations on days 17, 18 and 19, increased LH pulse frequency on day 18 and higher LH peaks during their oestrous cycles, though the pFF concentrations of oestradiol did not differ from those of control-fed gilts (Glasgow *et al.*, 1999; Ferguson *et al.*, 2007). Additionally, progesterone concentration was lower on day 13 of the oestrous cycle in high fibre-fed gilts and their hormone profiles displayed a lower base line progesterone and lower circulating progesterone concentrations (Glasgow *et al.*, 1999; Weaver *et al.*, 2013). The changes in the levels of these hormones may be integral to ascertaining

a mechanism of the diet on reproductive performance. For example, a high fibre diet is often associated with lowers circulating levels of cholesterol which may affect the process of steroidogenesis in the ovary, leading to the observed alterations in hormones (van Bennekum *et al.*, 2005; Venkatesan *et al.*, 2007). Alternatively, Ferguson *et al.*, (2007) and (Ashworth *et al.*, 2008) suggested that the decrease in circulating oestradiol may be due to binding of oestrogens to the fibre in the gut, and that this may lead to the reduction of the hormone's negative feedback effects on the hypothalamic-pituitary-ovarian (HPG) axis.

1.5.2. Feeding dietary fibre during gestation gave variable results and can be confounded by parity

The majority of studies that fed pigs a high fibre diet during different periods of gestation did not find any effect on reproductive parameters such as farrowing rate, the total number of pigs born, the number of pigs born alive, still born, mummified or litter size after cross fostering (McGlone and Fullwood, 2001; Darroch *et al.*, 2008; Renteria-Flores *et al.*, 2008; DeDecker *et al.*, 2014; Sun *et al.*, 2014). However, a couple of studies did observe alterations in litter size due to a gestational high fibre diet, though these are contradictory; high fibre-fed sows (fed one day after weaning until gestational day 109 of the following pregnancy) had fewer pigs born compared to sows fed a non-supplemented feed (Holt *et al.*, 2006). Conversely, high fibre-fed gilts fed during gestation as well as the weaning to oestrus interval had an increased number of total piglets born and number of live born piglets (van der Peet-Schwering *et al.*, 2003). These differences can be attributed to the different fibre sources and fibre contents used in the studies and the different periods the feed was allocated.

There are also inconsistencies in number of piglets weaned and piglet performance associated with dietary fibre during gestation. Some studies found that sows fed a high fibre diet farrowed and weaned more pigs per litter, and the total litter birth weight, piglet weight gain and weaning weights of the offspring were greater than control-fed sows (Guillemet *et al.*, 2007; Oliviero *et al.*, 2009; Quesnel *et al.*, 2009; Veum *et al.*, 2009; Peltoniemi *et al.*, 2010). However, other studies contradicted these findings as they found no treatment effect of different amounts of fibre on the number of pigs

weaned, litter weight at birth, average pig weight at birth, weaning weight or piglet growth rate (Matte *et al.*, 1994; Loisel *et al.*, 2013; Sun *et al.*, 2014). Additionally, one study found that sows fed increased dietary fibre from the day of breeding through to day 4 post-partum and at weaning, weaned lighter pigs than sows fed the control diet (Darroch *et al.*, 2008).

A couple of studies have also observed differences in the benefits of feeding a high fibre diet between different parities. In one study, sows fed increased dietary fibre during their first and second pregnancy farrowed more live pigs in the first parity whilst in the second parity, total litter weight was lower and the mean growth of individual pigs was slower in litters from high fibre sows (Matte *et al.*, 1994). In another study, within litter uniformity was significantly higher in high fibre-fed sows in the first parity whilst high fibre-fed sows had more live pigs and had higher litter weight in the second parity (Che *et al.*, 2011).

1.5.3. High fibre diets in other species

Interestingly, the effect of high fibre diets on fertility have not been assessed in most other domestic species. Studies that have assessed the effects of fibre diets focused on agricultural parameters such as growth, gut health and milk production, as opposed to reproductive parameters such as oocyte quality, ovulation and fertilisation rate, embryo survival and litter size (Dorshorst and Grummer, 2002; Broderick *et al.*, 2015; Castells *et al.*, 2015). However, there have been studies in hens whereby supplementation of rearing-feed with 5–15% whole flaxseed increased egg production (Scheideler and Froning, 1996). However, this increase in egg production was not observed when the diets were supplemented with corn dried distiller grains, wheat middlings, soybean hulls, cereal straw or sugar beet pulp (Roberts *et al.*, 2007; Guzmán *et al.*, 2016). Additionally, rabbit does fed diets supplemented with sugar beet pulp before mating did not have significantly different litter sizes compared to control-fed does (Martínez-Paredes *et al.*, 2012; Delgado *et al.*, 2018). However, feeding rabbit does rearing diets supplemented with defatted grapeseed meal resulted in detrimental effects, including significantly increased follicular apoptosis and significantly

decreased the proportion of MII oocytes and the viability of embryos (Arias-Álvarez *et al.*, 2010).

1.5.4. Digestion of high fibre diets and potential mechanisms of action

To hypothesise possible mechanisms towards the high fibre diet's effect on reproduction, it is important to understand the way in which the high fibre is digested in the pig. Dietary fibres are hydrolysed and metabolised by intestinal bacteria, which leads to the production of ATP, used for basal metabolism and growth of gut bacteria (Martinez-Puig *et al.*, 2003). Additionally, short-chain fatty acids, such as acetate, propionate and *n*-butyrate, as well as gases such as carbon dioxide, hydrogen and methane are also produced during this intestinal fermentation process (Bindelle *et al.*, 2008). These fatty acids can contribute to the pig's energy supply, but its contribution is considerably less than that of glucose following an equivalent intake of digestible starch (Noblet and Le Goff, 2001). Therefore, it is unlikely that the fermentation process that occurs is directly linked to the reproductive outcomes.

Fibres have many physical properties, including the ability to absorb and hold water, allowing different fibre types to influence digestion, satiety and feed transit time (Souza da Silva *et al.*, 2012). If satiety is heavily influenced by the fibre diet, it is possible that neurotransmitters involved in the regulation of food intake could be implicated. A prime example is neuropeptide Y (NPY), a neurotransmitter heavily involve in food intake as well as the secretion of gonadotrophin releasing hormone in the hypothalamus (Mcdonald, 1990; Wójcik-Gładysz and Polkowska, 2006).

However, the most promising indication of the physical properties of fibre influencing reproduction, is the ability of fibre to bind to oestrogens. Oestrogens are metabolised and conjugated in the liver, making them water soluble (Sher and Rahman, 2000). Half of the excess conjugated oestrogens in the plasma are then excreted via the kidney in urine whilst the other half is through the hepatic system as bile into the intestine (Sher and Rahman, 2000). However, most of these oestrogens are reabsorbed through the intestinal wall back into the liver (Sher and Rahman, 2000). *In vitro* studies have shown that oestradiol bind to various fibre types, whilst rat studies have shown that faecal excretion of free and unconjugated oestrogens was three times

higher just 24 hours after being fed a wheat bran based diet with an increased fibre content (Arts *et al.*, 1991a, b). Additionally, male rats injected with C^{14} labelled oestradiol and fed a high-fibre wheat bran diet excreted twice the amount of oestradiol during the first day after injection and after three weeks compared to rats fed low fibre diets (Arts *et al.*, 1992). Therefore, this binding of circulating oestrogens in the intestines lowers circulating levels in the animal which could influence their cycles.

1.6. General Hypothesis and Objectives

1.6.1. Hypothesis

The evidence suggests that a high fibre diet is most effective at enhancing reproductive performance when fed prior to ovulation as this is the period in which the ovarian follicule and the pFF inside can be susceptible to metabolic changes. Therefore, I hypothesised that follicular fluid composition was altered in response to the high fibre diet, which would then directly or indirectly lead to the improvements on oocyte maturation. This hypothesis is substantiated by the fact that the diet affects the early development of oocytes and blastocysts as opposed to ovulation and fertilisation rates.

1.6.2. Experimental and Research Objectives

There were two main objectives of this study were:

1) To establish whether a high fibre diet altered the molecular composition of pFF
2) To ascertain the molecular mechanisms involved in the effects of nutrition on porcine reproduction.

The first set of molecules targeted for analysis were proteins, given their important biological functions within the ovarian follicle. Therefore, a proteomic workflow was designed and implemented, and the subsequent direction of the study depended in part on the results of the proteomic analysis, described in chapter 2.

2. Proteomic Analysis of Porcine Follicular Fluid

2.1. Introduction and research aims

A high fibre diet is associated with several reproductive benefits, which begin at the cellular level with improved oocyte maturation (Ferguson *et al.*, 2003, 2004, 2006, 2007; Ashworth *et al.*, 2008). The proteins in porcine follicular fluid (pFF) perform several functions in the growth of the ovarian follicle and the maturation of the oocyte. Therefore, investigating the proteome of pFF to ascertain whether a high fibre diet affects its protein composition can give indications of any perturbation or enhancement of relevant molecular mechanisms involved in improved fertility.

The aim of this study was to identify whether a high fibre diet fed to pigs during their oestrous cycle altered the protein composition of their pFF, and whether these alterations were associated with later fertility. To do this, a proteomic study of pFF associated with different dietary regimes and *in vitro* fertilisation (IVF) outcome was carried out. However, like other biological fluids such as plasma and serum, pFF protein composition is dominated by a small number of proteins, such as albumin, transferrin, apolipoprotein A1 and fibrinogen (Bijttebier *et al.*, 2009; Sun *et al.*, 2011). Although these proteins are involved in mechanisms such as immune response and their expression can indicate perturbations in these mechanisms, they also mask the detection of other proteins present at lower concentrations (Bijttebier *et al.*, 2009; Sun *et al.*, 2011).

Therefore, the first step towards an accurate proteomic study was to establish the most appropriate preparatory techniques preceding mass spectromic detection. Two non-species-specific approaches were tested on spare pFF; these were the digestion-depletion method as described by Fonslow *et al.*, (2013) and the Proteominer Enrichment kit. With an optimised preparatory protocol, the main experimental aim was to carry out a proteomic analysis of pFF from control and high fibre-fed pigs to identify differentially proteins associated with pre-mating diet (**Table 2.1**). Additionally, within each dietary group, pFF from animals whose oocytes produced blastocysts after IVF were compared with pFF from animals whose oocytes did not

produce a blastocyst. These additional analyses were carried out to identify differentially expressed proteins associated with later fertility. The samples were obtained during a nutritional study previously carried out by Ashworth *et al.*, (2008), described in the Material and Methods section of this chapter.

Proteomic comparisons	What it will identify
All Control versus All High Fibre (All-C vs. All-HF)	Proteins associated with different nutritional regimens
Control No Blastocyst versus Control Blastocyst (C-No vs. C-Bl)	Proteins associated with later fertility
High Fibre No Blastocyst versus High Fibre Blastocyst (HF-No vs. HF-Bl)	

Table 2.1. List of proteomic comparisons and the rationale for making each comparison.

The proteomic analysis compared the protein composition of porcine follicular fluid (pFF) from pigs fed a control diet with pFF from pigs fed a high fibre diet, pFF from pigs fed a control diet whose oocytes produced blastocysts following *in vitro* fertilisation (IVF) with pFF from pigs fed a control diet whose oocytes did not produce blastocysts, and finally pFF from pigs fed a high fibre diet whose oocytes produced blastocysts following IVF with pFF from pigs fed a high fibre diet whose oocytes did not produce blastocysts.

2.2. Materials and Methods

2.2.1. Sample history

Sample collection was described in Ashworth *et al.*, (2008) and illustrated in **Figure 2.1**. Briefly, 40 Large White x Landrace gilts were fed either a control barley-based diet or a high fibre diet (50% unmolassed sugar beet pulp inclusion; 20 per feeding group) for the first 19 days of their third oestrous cycle. The gilts were then slaughtered on day 19 of their third oestrous cycle and oocytes and pFF were collected from the 16 largest ovarian follicles of each animal. The pFF was pooled within each animal to make two sets of pFF pools. The first pool (Pool 1) was made from equal volumes from each follicle, the volume being that of the smallest follicle of the 16. The second pool (Pool 2) was composed of the rest of the pFF from the remaining 15 follicles. The volume of each pool from each animal was recorded. The oocytes were matured *in vitro* in Tissue Culture Medium 199, supplemented with 0.5 µg/mL luteinisation hormone (LH) and follicle stimulating hormone and 10% of the animals' own pooled pFF before fertilisation. The resultant embryos were cultured in North Carolina State University Medium-23 (NCSU-23) medium for 6–7 days. Blastocysts that formed were fixed and stained with Hoescht 33258 and blastocyst cell numbers were recorded. Of the 20 animals fed the control diet, nine had oocytes that produced blastocysts and of the 20 animals fed the high fibre diet, six had oocytes that produced blastocysts. **Appendix 1** shows the animal numbers (codes) that were fed the high fibre and control diets and whether they had oocytes that produced blastocysts following IVF. The pFF samples were stored at -80 °C since 2002.

2.2.2. Sample preparation

For the optimisation of digestion-depletion and Proteominer enrichment, pools of spare pFF were made by combining 30 µl aliquots from "Pool 2" pFF of each animal. For the analysis of the proteomic experiment, the experimental pools made were all control (All-C), all high fibre (All-HF), control with no blastocyst (C-No), control with blastocyst (C-Bl), high fibre with no blastocyst (HF-No) and high fibre with blastocyst (HF-Bl), as shown in **Table 2.1**. All-C and All-HF pools were composed of 20 µl of

pFF from 12 animals per pool, whilst C-No, C-Bl, HF-No and HF-Bl pools were composed of 40 µl of pFF from six animals per pool (**Figure 2.2** and **Figure 2.3**). "Pool 1" pFF from the six animals with the largest pFF volume within each feeding group and IVF outcome were used to make the experiment pools (**Appendix 1**). As illustrated in **Figure 2.4**, two sets of pools ("Set 1" and "Set 2") of each of the six experimental pools were analysed in the proteomic workflow.

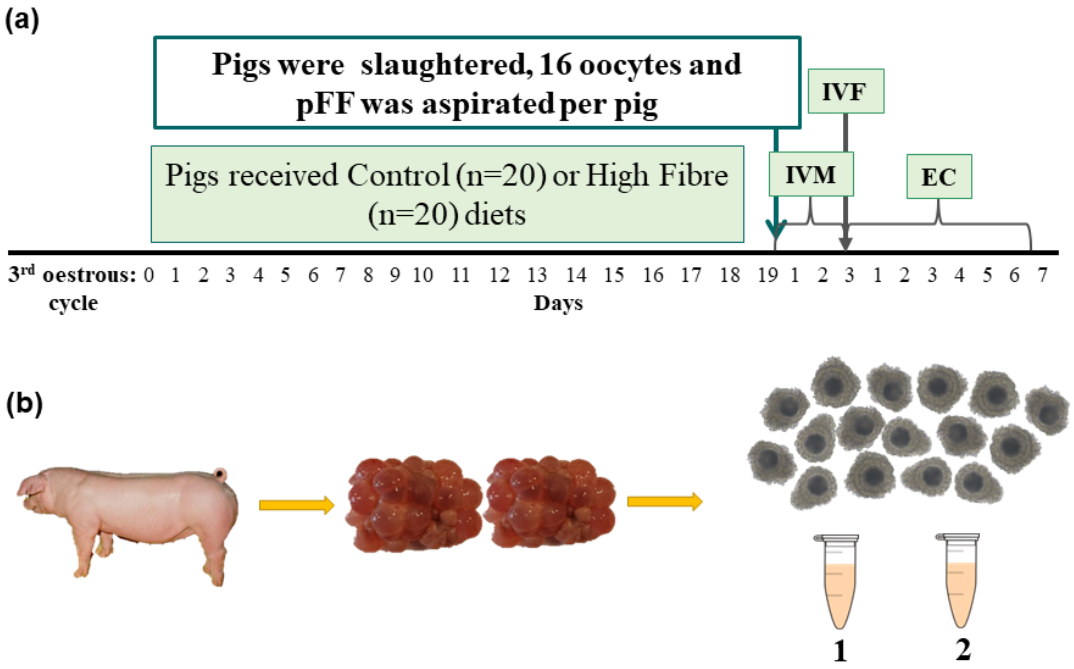


Figure 2.1. Schematics to illustrate the process of porcine follicular fluid (pFF) sample collection.

(a) Female pigs were fed either a control barley-based diet or a high fibre diet supplemented with 50% unmolassed sugar beet pulp during the first 19 days of their third oestrous cycle. On day 19, the pigs were slaughtered, and their ovaries were collected and aspirated of their oocytes. The oocytes were matured *in vitro* for two days in Tissue Culture Medium 199 supplemented with 10% of their own pooled pFF (Pool 1, see part b), before *in vitro* fertilisation and 7-day embryo culture. Animals were then categorised into whether their oocytes produced blastocysts. (b) After slaughter, during oocyte harvesting, the oocytes and associated follicular fluids were aspirated from the 16 largest follicles (the presumed ovulatory population) on each pair of ovaries from each animal. The fluids were pooled within animal to produce two pooled samples (Pool 1 and Pool 2); Pool 1 was composed of equal volumes of the 16 follicles (the volume being the smallest follicle of the 16), Pool 2 consisted of the remaining fluid.

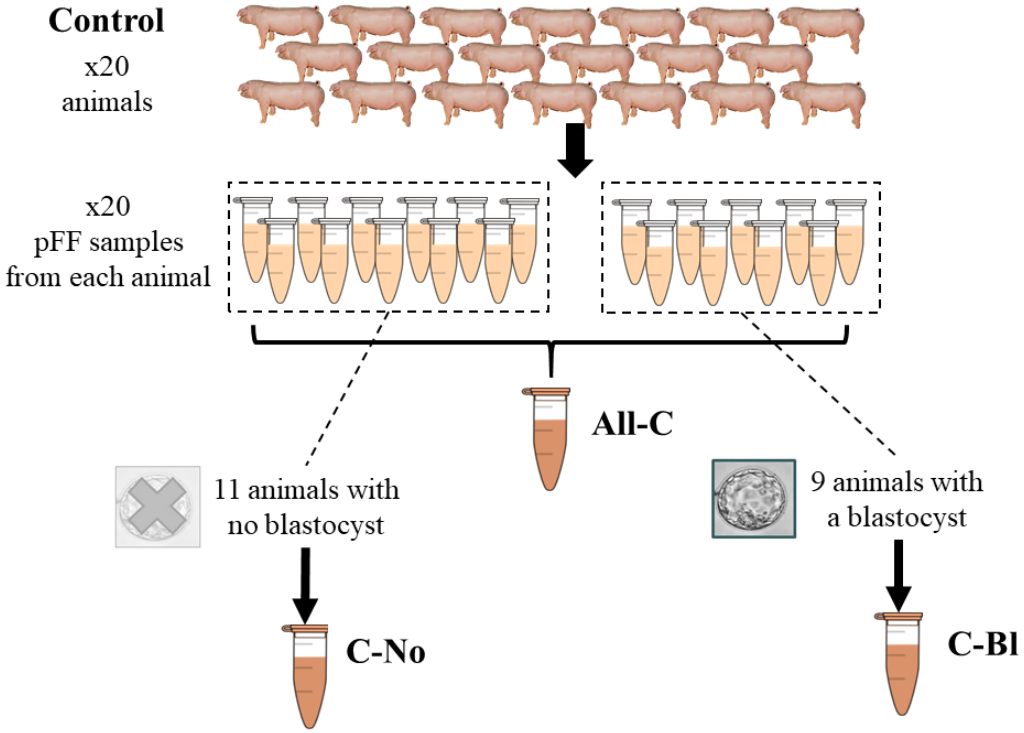


Figure 2.2. Schematic illustrating how the experimental pools in Table 2.1. were made from the control-fed animals.

Equal aliquots (20 µl) were taken from each porcine follicular fluid (pFF) sample from the 20 pigs fed the control diet to make an "All-C" experimental pool. Out of the 20 animals fed the control diet, 11 had oocytes that did not produce a blastocyst following *in vitro* maturation and fertilisation, and equal aliquots (40 µl) of pFF from these animals were combined to make the experimental pool "C-No". Nine animals did have oocytes that produced a blastocyst, and equal aliquots (40 µl) of the pFF samples from these animals were taken to produce the "C-Bl" experimental pool.

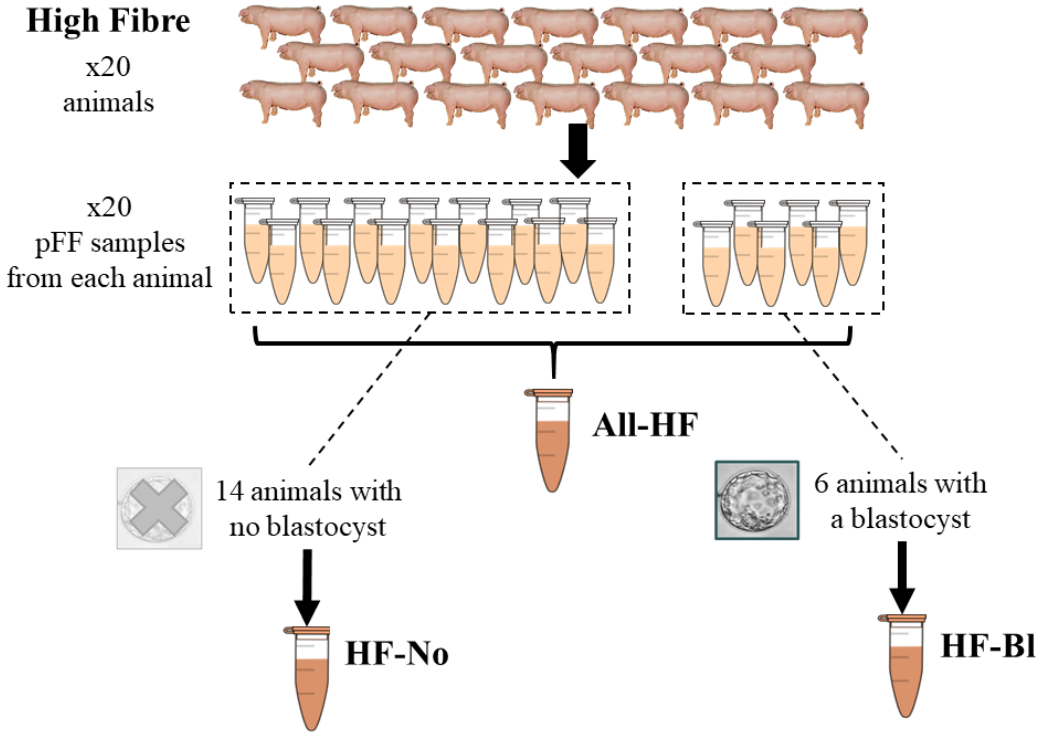


Figure 2.3. Schematic illustrating how the experimental pools in Table 2.1. were made from the high fibre-fed animals.

Equal aliquots (20 µl) were taken from each porcine follicular fluid (pFF) sample from the 20 pigs fed the high fibre diet to make an "All-HF" experimental pool. Out of the 20 animals fed the high fibre diet, 14 had oocytes that did not produce a blastocyst following *in vitro* maturation and fertilisation, and equal aliquots (40 µl) of pFF from these animals were combined to make the experimental pool "HF-No". Six animals did have oocytes that produced a blastocyst, and equal aliquots (40 µl) of the pFF samples from these animals were taken to produce the "HF-Bl" experimental pool.

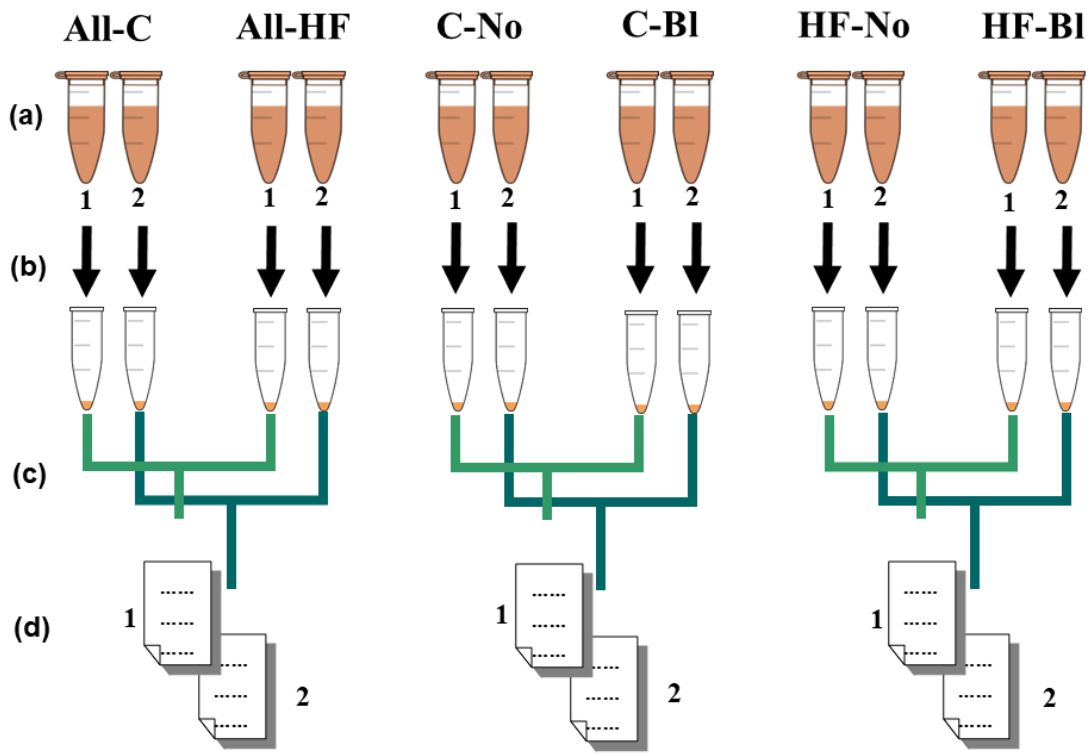


Figure 2.4. The proteomic workflow to detect differentially expressed proteins in pFF.

(a) Two replicates (Set 1 and Set 2) of each of the six pFF pools were created to provide replicates. (b) Each pool was depleted of abundant proteins with the Proteominer Enrichment kit. (c) The samples were digested into peptides, labelled, combined with the relevant partner. (d) The labelled peptides in each analysis were detected by tandem mass spectrometry to produce a list of proteins for Set 1 samples and Set 2 samples.

2.2.3. Protein assay

Protein assays were carried out using Pierce Bicinchoninic Acid Protein Assay kit – Reducing Agent Compatible. All reagents and materials were supplied by Thermo Fisher Scientific (Fountain Drive, Inchinnan, Renfrew, PA4 9RF, UK). Dilution series were carried out whereby crude pFF samples were diluted by 1/35, 1/70 and 1/140, digestion-depleted samples were diluted by 1/3, 1/6 and 1/9 and proteominer-depleted samples were diluted by 1/3. Diluents used included phosphate buffered saline (pH 7.4) for crude pFF and Proteominer enriched samples whilst 100 mM tris(hydroxyethylamine) was used for digestion-depleted samples. Absorbances were measured at 570 nm using the Perkin Elmer Precisely Spectrophotometer (1420 Multilabel Counter) with VICTOR^3 software/Wallac 1420 manager.

2.2.4. Reagents for digestion-depletion

All chemicals were provided by Sigma Aldrich (Second Avenue, Heatherhouse Industrial Estate, Irvine, KA12 8NB, UK) unless specified.

100 mM Tris(hydroxyethylamine)

For 500 mL, 6.057 g of solid tris(hydroxyethylamine) was dissolved in 100 mL deionised water (dH_2O). The pH of the buffer was adjusted to pH 8.5 using 12 M hydrogen chloride and/or 5 M sodium hydroxide (NaOH). The solution was then made up to 150 mL with dH_2O.

Denaturing solution

For 50 mL, 24.024 g of urea and 0.063 g tris(2-carboxyethyl)phosphine was dissolved in 25 mL tris(hydroxyethylamine) of pH 8.5. When the solid dissolved, the solution was made up to 50 mL with tris(hydroxyethylamine) of pH 8.5.

Rinsing solution

For 50 mL, 24.024 g of urea was dissolved in 25 mL tris(hydroxyethylamine) of pH 8.5. When the solid dissolved, the solution was made up to 50 mL.

100 mM Iodoacetamide solution

For 50 mL, 0.9246 g of iodoacetoamide was dissolved in 25 mL tris(hydroxyethylamine) of pH 8.5. When the solid dissolved, the solution was made up to 50 mL.

5 mM Calcium Chloride solution

For 200 mL, 0.111 g of calcium chloride ($CaCl_2$) was dissolved in 25 mL tris(hydroxyethylamine) of pH 8.5. When the solid dissolved, the solution was made up to 50 mL.

2.2.5. Reagents for denaturing and digesting proteins

All chemicals were provided by Sigma Aldrich unless specified.

50 mM HEPES Buffer

For 10 mL, 0.119 g of solid 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was dissolved in 5 mL of mass spectrometry grade deionised water ($MSdH_2O$, Thermo Fisher Scientific) and 60 µl of 5 M NaOH was added to adjust the pH to ~8.0 (confirmed with pH paper). The solution was then made up to 10 mL with $MSdH_2O$.

Lysis Buffer

For 3 mL, 1.44 g of solid proteomic grade urea was dissolved in 1.5 mL of 50 mM HEPES (pH 8.0) buffer and 30 µl of 10 % sodium dodecyl sulfate (SDS) was added to make a final concentration of 0.1% SDS. The solution was then made up to 3 mL with 50 mM HEPES (pH 8.0).

5 M Dithiothreitol

Solid dithiothreitol (DTT, Pierce, 20291) was resuspended in 100 µl of 50 mM HEPES (pH 8.0) buffer.

15 mM iodoacetoamide

One well of solid iodoacetoamide (Thermo, 90034) was resuspended in 132 µl of 50 mM HEPES (pH 8.0) buffer; 2.5 µl of this iodoacetoamide solution was added to both samples to give a final concentration of 375 mM.

2.2.6. Depletion of abundant proteins

Digestion-Depletion

The method was adapted from (Fonslow *et al.*, 2013). Briefly, for every 1 mg of protein in spare pFF, 250 µl of 8 M urea (Sigma-Aldrich), 100 mM Tris (hydroxyethylamine) pH 8.5 and 5 mM Tris(2-carboxyethyl)phosphine (Thermo Fisher Scientific, Auld Bond Road, Perth, PH1 3FX, UK) was added and left to incubate for 30 minutes at room temperature. The sample was incubated for 15 minutes in the dark with 10 mM iodoacetamide (Thermo Fisher Scientific) and then diluted with 100 mM tris(hydroxyethylamine) so that the concentration of urea became 2 M. Either trypsin/lys-C or trypsin (depending on the experiment) in 5 M calcium chloride (Sigma-Aldrich) was then added to the sample to make the final concentration of calcium chloride 1 M. Spare pFF was treated with different amounts of protease. For the initial experiment, 20 ng, 30 ng and 40 ng of both trypsin/lys-C and trypsin were added to 1 mg of spare pFF. The second experiment involved the addition of 40 ng, 120 ng and 360 ng of trypsin to 1 mg of spare pFF as the previous optimisation experiment was not reproducible. After the addition of protease, the sample was incubated for 12 hours at 37 ºC. Digests were transferred into Amicon Ultra-4, Ultracel 10K 10,000 kDa molecular weight cut off centrifugal filters (Millipore, Suite 21, Building 6, Croxley Green, Watford, WD18 8YH, UK) and the filters were spun at 2,500 xg for 30 minutes at 4 ºC until 100–200 µl remained in the filter. The filter was rinsed with 250 µl of 8 M urea (Sigma-Aldrich), 100 mM Tris(hydroxyethylamine) pH 8.5 and the urea was diluted to 2 M with 750 µl of 100 mM Tris(hydroxyethylamine). The filters were spun again at 2,500 xg for 30 minutes at 4 ºC until 100–200 µl remained in the filter. The 150 µl of depleted sample left on the filter was taken and combined.

Proteominer Protein Enrichment kit

Enrichment of low abundance proteins was carried out as per the instructions provided in the Proteominer small-capacity protein enrichment kit (Bio-Rad, Dryden Vale, Loanhead, EH20 9HN, UK). Briefly, the storage solution was removed from the columns by spinning at 1000 xg for 1 minute. The columns were prepared by washing them with 200 µl of Wash Buffer three times, rotating the column for 5 minutes during each wash and centrifuging at 1000 xg for 1 minute after each wash. Columns were loaded with 10 mg of undepleted pFF and incubated at room temperature with shaking for 2 hours. The columns were centrifuged at 1000 xg for 1 minute and washed with 200 µl of Wash Buffer three times, rotating the column for 5 minutes during each wash and centrifuging at 1000 xg for 1 minute after each wash. The column was rinsed by rotating the column for 1 minute with 200 µl dH_2O which was then removed by centrifuging at 1000 xg for 1 minute. Low abundance proteins were eluted with 3 x 20 µl Rehydrated Elution Reagent, vortexing for 5 seconds after each aliquot and incubated for 15 minutes at room temperature, vortexing every 2.5 minutes. Elutions were collected by centrifuging at 1000 xg for 1 minute after each 20 µl aliquot and were stored at -20 °C.

The Proteominer protein enrichment kit from Bio-Rad involves the use of a column containing a combinatorial library of 64 x 10^6 hexapeptide beads that uniquely bind to proteins (Paulus *et al.*, 2009). The nature of the binding between the proteins and peptides is not completely known, other than that the technology works by exploiting the limited number of binding sites on a protein, meaning that proteins of high abundance quickly reach their bead capacity and excess abundant proteins are able to be washed out (Paulus *et al.*, 2009).

2.2.7. Acetone precipitation

For a given volume of sample, six times that volume of -20 °C acetone (SigmaAldrich) was added to the sample and gently vortexed. Samples were then incubated overnight at -20 °C and the proteins were pelleted by spinning at 15,700 xg for 10 minutes. The supernatant was removed, and 1 mL of ice cold 90% acetone was added to the sample,

vortexed and centrifuged for 5 minutes at 15,700 xg. All the supernatant was removed, and the protein pellet was air dried for one hour before being resuspended in the appropriate buffer.

2.2.8. One-dimensional polyacrylamide gel electrophoresis

All reagents and materials were supplied by Life Technologies (Birchwood, Warrington, WA3 7QH, UK) unless specified. Wells contained 7.5 µg of protein sample and 25% SDS sample buffer. Blank wells contained 25% SDS sample buffer and 75% dH_2O. Samples and blanks were heated to 70 ºC for 10 minutes. The onedimensional polyacrylamide gel electrophoresis (1-D PAGE) was carried out using NuPAGE Novex Tris-Acetate gels, an X-Cell Surelock Mini Cell and running buffer composed of 950 mL dH_2O and 50 mL of NuPAGE MOPS Running Buffer. Prosieve Color Protein Markers (Lonza, Wheldon Road, Castleford, WF10 2JT, UK) was used as the ladder. Electrophoresis was carried out at 200 V for 50 minutes. The gel was released from the cassette and the lanes and foot of the gel were cut off with a gel knife. Gels were washed with 50 mL of dH_2O for 6 x 2.5 minutes, stained with 20 mL of Simply Blue Safe Stain for 1 hour and washed with 50 mL of dH_2O for 2 x 30 minutes with stirring. Gels were then incubated overnight with 50 mL of dH_2O and 10 mL 20% sodium chloride (Thermo Fisher Scientific) with stirring. Scanning of gels was carried out with the Odessey scanner and Image Studio software (Licor Biosciences, St. John's Innovation Centre, Cowley Road, Cambridge, CB4 0WS, UK).

2.2.9. Complete denature and digestion

The samples were treated with a solution of 8 M urea, 0.1% SDS and 50 mM HEPES (pH 8.0, Sigma-Aldrich) to make a total volume of 50 µl. Proteins were reduced with DTT in 50 mM HEPES (pH 8) to a final concentration of 10 mM, mixed by vortex and then spun down at 13,000 xg for 1 minute and incubated for 1 hour at 37 °C with shaking. Cysteine residues were alkylated with 15 mM iodoacetoamide (IAA, Thermo Fisher Scientific) in 5 mM HEPES (pH 8), mixed by vortex and then spun down at 13,000 xg and incubated for 45 minutes in the dark at room temperature. The excess iodoacetoamide was quenched with 1.1 µl DTT for 20 min at room temperature. Reduced and alkylated proteins were digested with trypsin/lys-C mix (Promega,

Enterprise Road, Chilworth, Southampton, SO16 7NS, UK) and resuspended in 5 mM HEPES (pH 8) in an enzyme to protein ratio of 1:20. Samples were incubated at 37 °C for 3 hours, diluted with 450 µl of 5 mM HEPES and incubated overnight at 37 °C. For digestion-depleted samples 200 µg were analysed for a mass spectrometry analysis and Proteominer enriched samples had between 20–95 µg of protein.

2.2.10. Dimethylation labelling and solid phase extraction

All reagents and materials were supplied by Sigma-Aldrich unless specified. Dimethylation labelling was carried out in conjunction with solid phase extraction, using 50 mg Sep-Pak Vac Icc (Waters, Centennial Avenue, Elstree, Borehamwood, WD6 3SZ, UK). All reagents for solid phase extraction were made using MS-dH_2O, Thermo Fisher Scientific and the protocol was adapted from Wilson-Grady et al. (2013). Briefly, samples were cooled on ice and acidified with 10% trifluoroacetic acid (TFA, Thermo Fisher Scientific) to make a final concentration of 0.4% TFA. Cartridges were washed with 1.5 mL of acetonitrile (ACN, Thermo Fisher Scientific), 1.5 mL of 50% ACN/0.1% TFA and 1.5 mL 0.1% TFA. Samples were loaded, and the sample filtrates were collected and reloaded onto the columns. The cartridges were washed with 1.5 mL of 0.1% TFA and 3 mL of 0.25 M 2-(*N*-morpholino) ethanesulfonic acid (MES, pH 5.5). Samples were labelled by passing 5 mL of either "light" label (0.4% formaldehyde, 0.25 M MES pH 5.5, 60 mM sodium cyanoborohydride) or "heavy" label (0.4% D_2-formaldehyde, 0.25 M MES pH 5.5, 60 mM sodium cyanoborohydride). For Set 1, peptides from All-C, C-Bl and HF-Bl were labelled with the light label and All-HF, C-No and HF-No were labelled with the heavy label. For Set 2 the labels were swapped round so that peptides from All-C, C-Bl and HF-Bl were labelled with the heavy label and All-HF, Con-No and HF-No were labelled with the light label. Cartridges were then washed with 1.5 mL of 0.1 TFA and 250 µl of 0.5% acetic acid (AcOH). Peptides were eluted from the column with 1.5 mL of 80% ACN/0.5% AcOH. Light and heavy labelled samples were combined and dried by rotary drying using the miVac DNA Concentrator GeneVac Speed Vac (Thermo Fisher Scientific).

2.2.11. Liquid chromatography tandem mass spectrometry

This section was carried out by Dr Dominic Kurian. All reagents and materials were supplied by Fisher Scientific unless specified. The protein digests (labelled or unlabelled) were first fractionated by Strong Cation Exchange (SCX) chromatography on a Polysulfoethyl A column (PolyLC, 9151 Rumsey Road, Suite 180, Columbia, Maryland, 21045, USA) using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific). This separated peptides into fractions according to charge and hydrophobicity which was visualised with Chromeleon software. Fractions were then dried using the miVac DNA Concentrator GeneVac Speed Vac, re-suspended in 50% methanol, dried and re-suspended in 50% methanol and dried again. Each fraction was then re-suspended in 0.2% Optima LC/MS grade formic acid and 4% Optima LC/MS grade ACN, made up in Optima LC/MS grade water. Each fraction of peptides was loaded on to an Acclaim PepMap100, C18, 3 μm, 100 Å, 75 μm i.d. × 50 cm column using RSLC Ultimate nano LC System (Dionex, Albany Court, Albany Road, Albany Park, Camberley, Surrey, GU16 7QL, UK). The peptides eluted with reversed-phase chromatography were analysed by a micrOTOF QII Mass spectrometer (Bruker, Banner Lane, Coventry, CV4 9GH, UK). Raw mass spectral data were processed with data analysis software (Bruker) to generate peaks lists which were searched against Uniprot Human protein sequences using Mascot 2.4 (Matrix Sciences, Baker Street, Marylebone, London, W1U 7GB, UK). Protein identifications were filtered using both a 1% false discovery threshold and a requirement for two or more peptides using Proteinscape 3.0 software (Bruker). Protein quantification was performed using WARP LC 1.3 software (Bruker).

2.2.12. Ingenuity pathway analysis

Each analysis was searched through Uniprot to obtain a dataset detailing accession number related to uniprot databases (Swissprot and TrEMBL). Other information given includes protein name and molecular weight and isoelectric point, the number of alternative proteins that peptide signals could be assigned to, mascot scores, the number of peptides for that protein, the sequence coverage of each protein, root mean square of mass tolerance, rank, the heavy/light ratio, the number of heavy/light label

ratios, and the coefficient variation of peptides (%). Proteins detected with heavy label/light label ratios higher than 1.25 and lower than 0.8 were regarded as differentially expressed between heavy labelled and light labelled samples.

Differentially expressed proteins were submitted into Ingenuity Pathway Analysis (IPA) software. For this, the Uniprot Accession Numbers (from Pig) were converted to Human Ensembl codes that were compatible with the software. This is because the IPA software does not include the porcine proteome, only the human and rodent genome. A database was downloaded listing the Pig Ensemble Gene ID, Pig Ensembl Transcript ID, Pig Description, Pig Uniprot/Swissprot code, pig Uniprot/TrEMBl code, corresponding Human Ensembl Gene ID, corresponding Human Ensembl Transcript ID and corresponding Human Description. The database was obtained from www.emsembl.org/biomart (accessed on January 2015). Pig Uniprot accession numbers were matched to their corresponding human Ensembl gene codes counterparts.

Some Ensembl codes were derived from the Swissprot/Trembl Uniprot Accession numbers whilst others were derived from the protein name. Some were derived from searching the Uniprot number on www.uniprot.org (accessed on January 2015), clicking on "Genome annotation databases", "Gene ID" and obtaining the Ensembl code for Pig. This process was automated using a Bash script carried out by Rodrigo Bacigalupe (on Linux). The pig ensembl codes were then matched with their counterpart human codes. A different database which was also obtained from www.emsembl.org/biomart (accessed on January 2015) was used for this. The database only contained the pig gene Ensembl ID, pig transcript Ensembl ID, Human Ensembl ID and pig protein Ensembl ID.

The human Ensembl codes relating to proteins with heavy/light ratios higher than 1.25 or lower than 0.8 were uploaded into the IPA online software, available on www.ingenuity.com, which was accessed from January to February 2015. From the IPA interface, the results generated "Canonical Pathways", "Upstream Analysis", "Disease and Functions", "Regulator Effects", "Networks, Lists", "My Pathways" and "Molecules" tabs. For each analysis, the top 15 canonical pathways were identified.

2.2.13. Candidate selection

IPA was used to identify the top canonical pathways the differentially expressed proteins were associated with. The top 15 pathways in each analysis was identified and once the pathways most commonly in the top 15 were identified, proteins that were differentially expressed in at least three analyses were highlighted as potential candidates. Additionally, in all the datasets, the proteins with the greatest expression levels (either highest or lowest heavy/light ratios) and with mascot scores of greater than 1500 were identified. Identifying the proteins that were the most differentially expressed and had the highest probability of the experimental data matching correctly with the database sequence (Mascot score), provides confidence in selecting the candidates most likely to validate.

2.3. Results

2.3.1. Comparisons of the most abundant proteins in depleted pFF to optimise the depletion method

Before carrying out the proteomic study, it was important to identify the most appropriate depletion method preceding mass spectrometry analysis. The depletion of abundant proteins would allow for the detection of low abundance proteins which may be of potential interest. The first method tested was the digestion-depletion method which does not utilise immunodepletion technology and is based on a recently published study (Fonslow *et al.*, 2013). The method works on the principle that the rate of hydrolysis of a substrate is proportional to its starting concentration MichaelisMenton kinetics, (Johnson and Goody, 2011). Therefore, when added to a sample, a protease at low concentration should selectively digest the proteins of highest abundance in a sample. The addition of trypsin to pFF effectively digested the proteins in the sample (**Figure 2.5**). As part of the optimisation process, the effect of different amounts of protease and which protease mixture was used was analysed. The 1-D PAGE showed no difference between using trypsin or trypsin/lys-C and that the amount of each protease added did not have an effect on the depletion (**Figure 2.6a**). Additionally, the previous observation in **Figure 2.5** was not replicated as the sample was not depleted after the addition of 40 ng trypsin/lys-C per mg protein which was used in that initial experiment. The level of digestion-depletion previously observed was only seen again with the addition of 360 ng trypsin (**Figure 2.6b**). These results provided little confidence in the efficiency and reliability of the digestion-depletion method and therefore an alternative method was considered.

The Proteominer Enrichment kit was tested and compared against the digestiondepletion method. The Proteominer enrichment column contains hexapeptide beads with a limited number of unique binding sites for different proteins so that proteins of high abundance quickly reach their bead capacity and the excess abundant proteins are able to be washed out (Paulus *et al.*, 2009). **Figure 2.7a** shows the 1D-PAGE of undepleted pFF, pFF enriched with the Proteominer kit and pFF treated with 360 ng trypsin per mg of protein. An initial concern with the digestion-depletion

method was that it was not known whether the bands detected on the gel were low abundance proteins or simply digests from high abundance proteins. The Proteominer lane in **Figure 2.7a** shows a wider spread of bands compared to the trypsin lane, suggesting that digestion-depletion targets larger proteins whereas the Proteominer retains some proteins of high molecular weight. The Proteominer kit also shows a good level of reproducibility as shown in the 1D-PAGE of two different enriched pFF samples in **Figure 2.7b**.

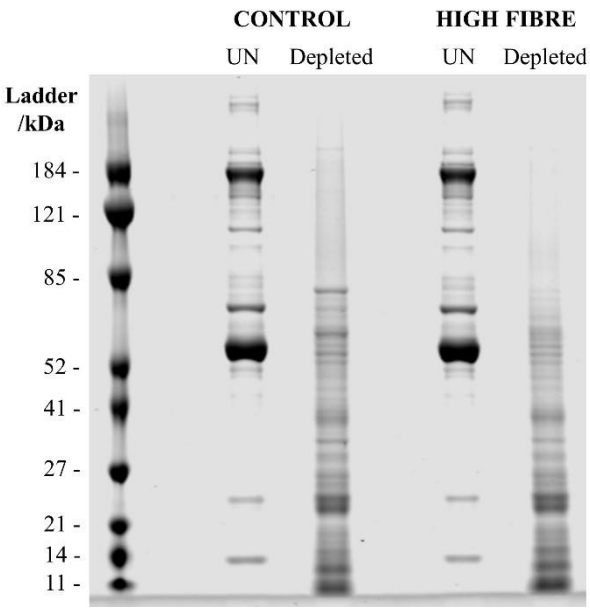


Figure 2.5. Electrophoresis gel with undepleted and trypsin-digested spare pFF samples.

Lanes in order are as follows; ProSieve Color Protein Marker (kDa), undepleted (UN)-control, Depleted-control, UN-high fibre and depleted-high fibre. Depleted samples were treated with 20 ng of trypsin for every mg of protein in the fluid. There were noticeable reductions in abundant proteins such as albumin (66 kDA) in the depleted samples in both control and high fibre groups, compared to the undepleted samples. Proteins were stained with Simply Blue Safe Stain. Ladder was Prosieve Color Protein Markers.

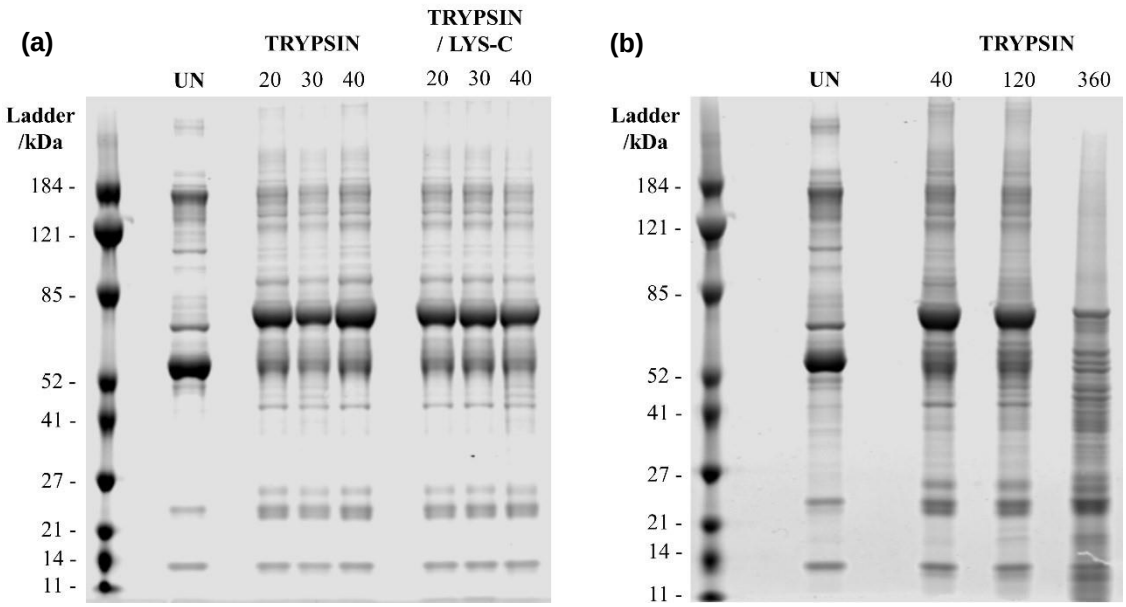


Figure 2.6. Electrophoresis gels with spare pFF samples that were digestion-depleted with different amounts of trypsin.

(a) Spare pFF which was either undepleted (UN) or treated with 20 ng, 30 ng and 40 ng trypsin and trypsin/lys-C; (b) spare pFF which was either undepleted (UN) or treated with 40 ng, 120 ng and 360 ng trypsin for every mg of protein in the fluid. All the numbers at the top of the lanes on both gels correspond to ng of trypsin. There were no observable differences in the level of digestion between 20, 30 and 40 ng of trypsin or trypsin/lys-C. Additionally, there were no differences in the level of digestion between the two enzyme mixtures. A noticeable difference was only observed when 360 ng of trypsin was added to pFF compared to 40 and 120 ng. Proteins were stained with Simply Blue Safe Stain. Ladder was Prosieve Color Protein Markers.

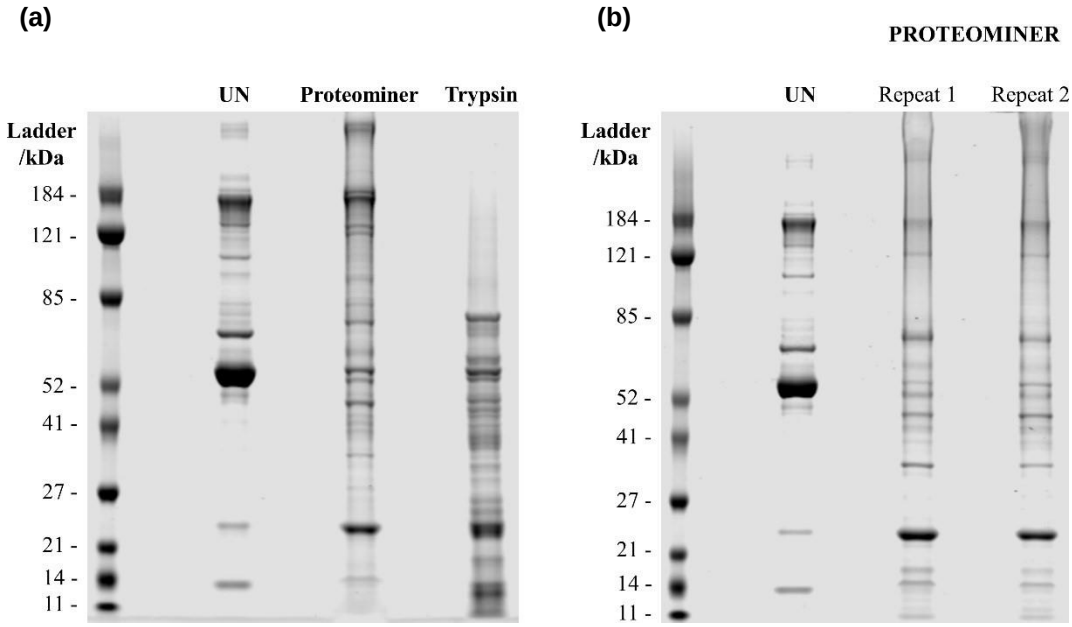


Figure 2.7. Electrophoresis gels comparing trypsin digestion with Proteominer enrichement.

The gel in (a) compares pFF composition of undepleted (UN) spare pFF, pFF enriched with the Proteominer kit and pFF digestion-depleted with 360 ng trypsin. In both Proteominer and trypsin treated samples, the masking effect of abundant proteins such as albumin was reduced. However, the distribution of molecular weights was greater in Proteominer samples compared to trypsin samples. In the trypsin samples, only proteins and peptides of low molecular weight were observed. The gel in (b) analyses the reproducibility of Proteominer enrichment kit by carrying out two more enrichments on spare pFF samples. This gel shows a high amount of reproducibility between the two repeats. Proteins were stained with Simply Blue Safe Stain. Ladder was Prosieve Color Protein Markers.

2.3.2. Electrophoresis gels to confirm depletion of abundant proteins in the experimental follicular fluid samples and the detection of proteins by liquid chromatography tandem mass spectrometry

Once an appropriate depletion method was identified, the experimental pools were depleted of abundant proteins as confirmed by 1D PAGE gels (**Figure 2.8a** for Set 1 and **Figure 2.8b** and for Set 2). Following depletion and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, identified peptides were quantified and filtered using a 1% false discovery threshold and a requirement for two or more peptides (**Table 2.2**). In Set 1 dataset, 768, 754 and 900 proteins were detected in total in All-C versus All-HF, C-No versus C-Bl and HF-No versus HF-Bl analyses respectively (**Supplementary Tables 1–3** on disc). On average, 52.6%±1.7 (mean±SEM) of the total proteins detected were detected with at least two peptides. In Set 2 datasets, 1397, 1485 and 1385 proteins were detected in total in AllC vs. All-HF, C-No vs. C-Bl and HF-No vs. HF-Bl analyses, respectively (**Supplementary Tables 4–6** on disc). On average, 64.5%±0.6 (mean±SEM) of the total proteins detected were detected with at least two peptides. There were more proteins unique to Set 2 datasets than there were proteins unique to Set 1 datasets or present in both (**Figure 2.9**).

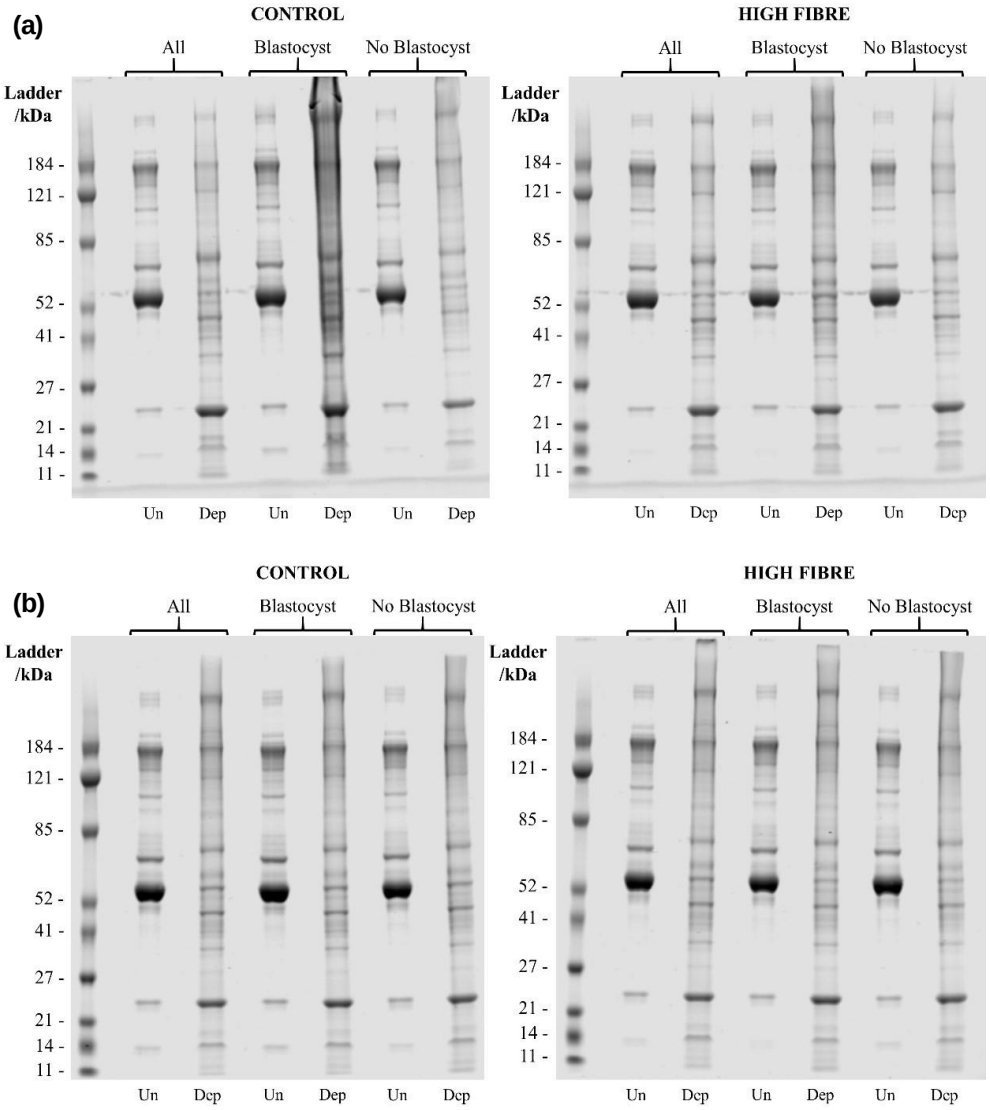


Figure 2.8. Electrophoresis gels with pooled pFF samples confirming Proteominer enrichment.

Electrophoresis gels for (a) Set 1 and (b) Set 2 pooled samples. Gels on the left show all the pFF samples from control-fed pigs and gels on the right show the pFF samples from high fibrefed pigs. On each gel, the first two lanes are samples from all the control or high fibre-fed pigs, followed by samples from pigs whose oocytes produced blastocysts and then samples from pigs whose oocytes did not produce blastocysts. Within each pair of samples, the left show the un-depleted sample (Un) whilst the right show the depleted/Proteominer enriched sample (Dep). These gels confirmed the enrichment of low abundance proteins in the samples and indicated an acceptable level of reproducibility between sample pools. Ladders were Prosieve Color Protei Markers.

Analysis	Set	Total number of proteins detected	Proteins with at least 2 peptides (%)*
All-C vs. All-HF	1	768	429 (55.9)
	2	1397	886 (63.4)
C-No vs. C-Bl	1	754	389 (51.6)
	2	1485	960 (64.6)
HF-No vs. HF-Bl	1	900	455 (50.6)
	2	1385	906 (65.4)

Table 2.2. Total number of proteins detected in the proteomic analyses.

*Percentage of proteins with adequate number of peptides from the total number of proteins detected. On average, nearly twice as many proteins were detected in Set 2 datasets compared to Set 1. Additionally, a greater percentage of Set 2 proteins were sufficiently labelled (64.7% on average) compared to Set 1 proteins (52.9% on average). Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst.

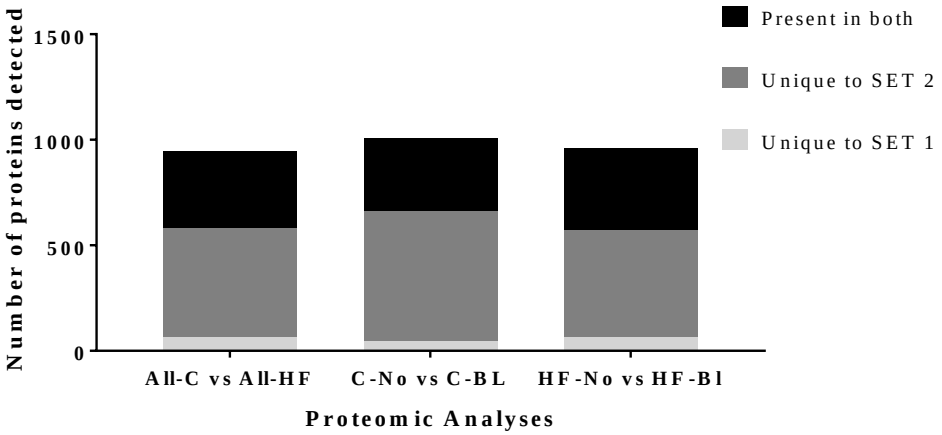


Figure 2.9. Bar chart illustrating the efficiency of protein detection between Set 1 and Set 2 analyses.

Stacked bar chart displaying the proportion of proteins within each analysis that were detected in both Set 1 data and Set 2 data, only Set 1 data and only Set 2 data. The majority of proteins detected were detected in Set 2 analyses. However, a large number of proteins were also present in both datasets and only a small number were unique to Set 1 datasets. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst.

2.3.3. Identification of differentially expressed proteins

Once the proteins with the sufficient number of detected peptides were filtered for, the proteins which were differentially expressed were identified. The differentially expressed proteins were those that had heavy/light ratios of higher than 1.25 or lower than 0.8. This study revealed several differentially expressed proteins within each analysis. In Set 1 and Set 2 analyses respectively, 277 and 179 proteins were differentially expressed between All-C and All-HF samples, 196 and 477 proteins were differentially expressed between C-No and C-Bl samples and 357 and 388 proteins were differentially expressed between HF-No and HF-Bl samples.

There were significant differences between the differentially expressed proteins of Set 1 and Set 2 results. In all of the analyses, the heavy/light ratios of Set 2 data have a more normal distribution compared to the heavy/light ratios of Set 1 data (**Figure 2.10**). Additionally, Set 2 was more discriminating in assigning differential expression, with a lower proportion of proteins outside of the cut-off points (dashed lines in the histograms of **Figure 2.10**. The heavy/light ratios of the proteins between Set 1 and Set 2 results were also not comparable (**Figure 2.11**).

Venn diagrams illustrate the proportion of differentially expressed proteins common to each analysis for Set 1 (**Figure 2.12a**) and Set 2 (**Figure 2.12b**) datasets; when the proteins compared were detected in all three lists. In Set 1, the majority of differentially expressed proteins (65.3%) were detected in the HF-No vs. HF-Bl analyses. This comparison also had the largest percentage of unique differentially expressed proteins (28.6%). The comparisons that shared the largest percentage of differentially expressed proteins were All-C vs. All-HF and HF-No vs. HF-Bl (15.9%). In Set 2, the majority of differentially expressed proteins (61.8%) were detected in the C-No vs. CBl analyses. This analysis also had the largest proportion of unique differentially expressed proteins (36%). Here, the vast majority of proteins were shifted to the left of the histogram (shown in **Figure 2.10d**), which corresponds to an increased expression of proteins in C-Bl samples. The analyses that shared the largest percentage of differentially expressed proteins were C-No vs. C-Bl and HF-No vs. HF-Bl (15.7%).

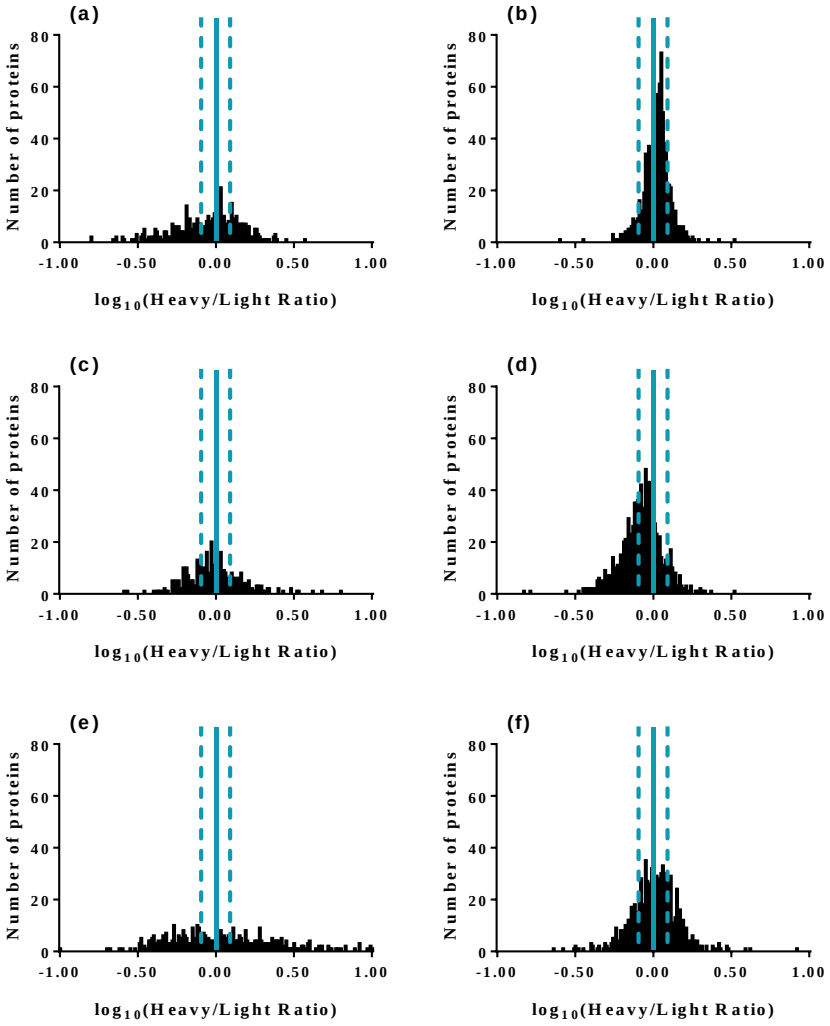


Figure 2.10. Histograms of the (log^{10}) heavy/light ratios for each analysis.

Histograms on the left and on the right are from Set 1 and Set 2 data respectively. Anything with a ratio higher than 1.25 or lower than 0.8 was considered to be a differentially expressed protein. The solid green line represents a heavy/light ratio of zero, which indicated no difference in expression. The green dashed lines show these cut-off points (log^{10} of 1.25 and 0.8) and outliers of these points are considered to be differentially expressed. Each analysis was represented; (a) (b) All-C vs. All-HF, (c) (d) C-No vs. C-Bl, (e) (f) HF-No vs. HF-Bl. Overall, Set 2 ratios have a more normalised distribution and are more discriminating in isolating differentially expressed proteins. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst.

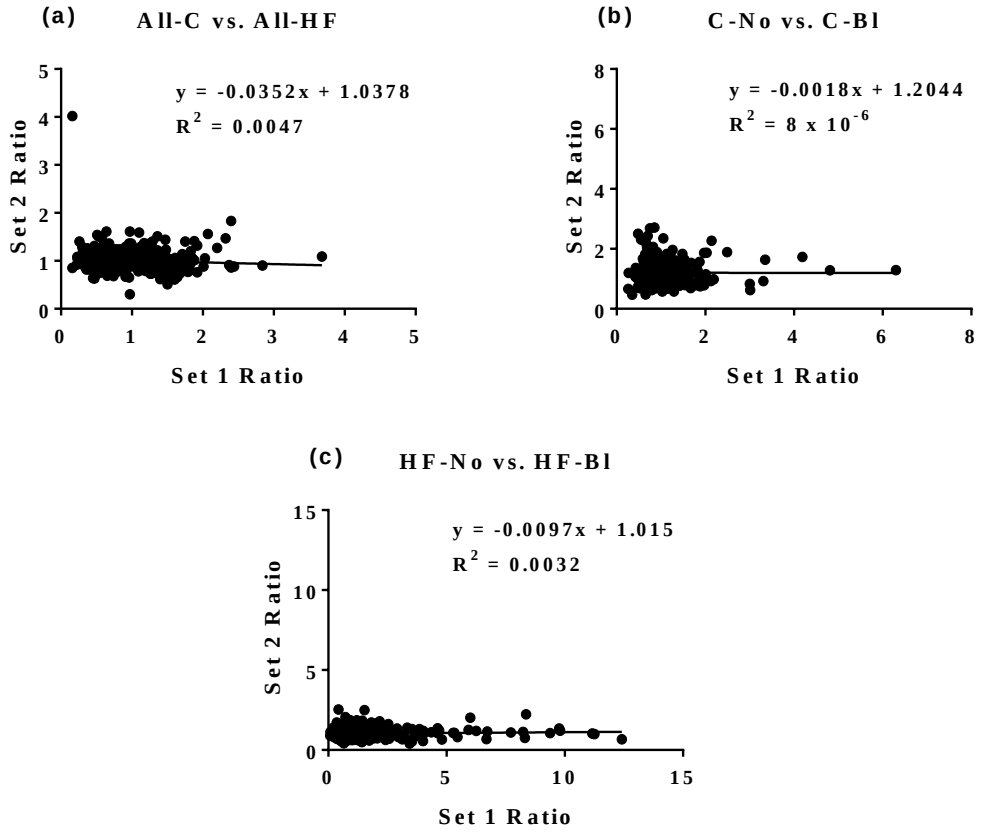
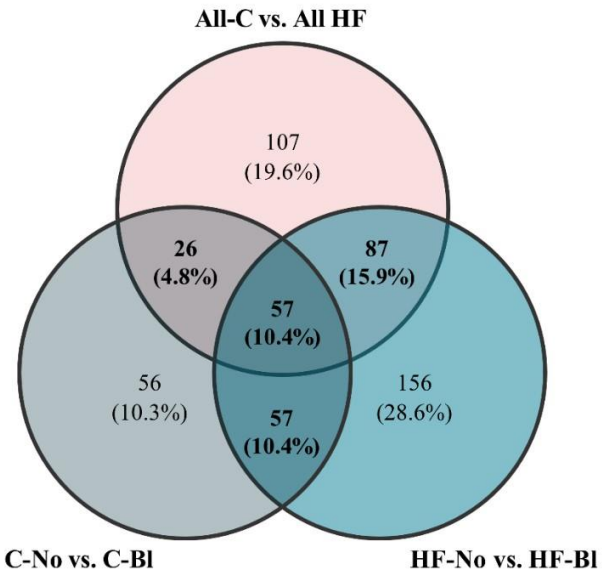


Figure 2.11. Scatter plots comparing the ratios from Set 1 and Set 2 analysis.

The scatter plots illustrate the heavy/light ratios of all the proteins detected in both Set 1 and Set 2 analyses; (a) All-C vs. All-HF, (b) C-No vs. C-Bl and (c) HF-No vs. HF-Bl. The x-axes show the Set 1 ratios whilst the y-axes show the Set 2 ratios. Theoretically, if the data between the two sets were exactly the same, the intercept would be 0, the coefficient in front of x would be 1, the R^2 value would be 1 and the line of best fit would have a 45 ° angle. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst.

(a)



(b)

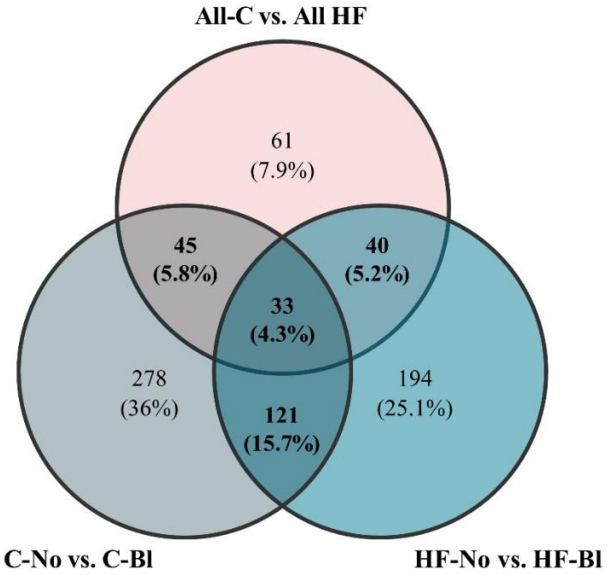


Figure 2.12. Venn diagrams of Set 1 and Set 2 differentially expressed proteins in each analysis.

In Set 1 data (a), the HF-No vs. HF-Bl analysis had most differentially expressed proteins followed by the All-C vs. All-HF analysis. The majority of the differentially expressed proteins were only differentially expressed in these two analyses. In Set 2 (b), the C-No vs. C-Bl analysis had most differentially expressed proteins followed by HF-No vs. HF-Bl. These two analyses also share a large number (121, 15.7%) of differentially expressed proteins. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst.

2.3.4. Top canonical pathways identified by IPA and selection of candidates for validation

In nearly all the analyses in Set 1 and Set 2 databases, IPA analysis identified similar top canonical pathways, but in slightly different orders. These molecular pathways included acute phase response signalling, liver X receptor/retinoid X receptor (LXR/RXR) activation, farnesoid X receptor (FXR)/RXR activation, coagulation system, complement system and intrinsic and extrinsic prothrombin activation (**Table 2.3** and **Table 2.4**). For selecting candidates for validation, it was important to identify molecules with potential biological significance. Using the several IPA parameters available, 11 molecules were selected as candidates for validation that were involved in/with the multiple canonical pathways detected. This was achieved by narrowing down the number of important pathways from 15 to four; the four canonical pathways which consistently appeared at the top of the lists of top canonical pathways in **Table 2.3** and **Table 2.4** were acute phase response signalling (**Table 2.5**), coagulation system (**Table 2.6**), FXR/RXR activation (**Table 2.7**) and LRX/RXR activation (**Table 2.8)**. The next step was to identify the proteins in those pathways that were differentially expressed in the most number of analyses. The proteins that were differentially expressed in at least three analyses are in bold in **Table 2.5** to **2.8**. In addition to this, **Table 2.9**shows the proteins in all the datasets with the greatest expression levels (either highest or lowest heavy/light ratios) and with mascot scores of greater than 1500. These proteins were identified as being the most differentially expressed and therefore would most likely to validate. Using a combination of the number of analyses the candidates were differentially expressed in, the heavy/light ratios themselves and the mascot scores, a sub-selection of 11 candidates were chosen for validation (**Table 2.10**). These candidates were apolipoprotein A4, apolipoprotein E, apolipoprotein M, ceruloplasmin, complement component 4 binding protein α, clusterin, inter-α-trypsin heavy chain H1, fibrinogen β, lipopolysaccharide binding protein, plasminogen, tropomyosin 1.

All-C vs. All-HF	C-No vs. C-Bl	HF-No vs. HF-Bl
Acute Phase Response Signalling	LXR/RXR Activation	Acute Phase Response Signalling
LXR/RXR Activation	FXR/RXR Activation	LXR/RXR Activation
FXR/RXR Activation	Acute Phase Response Signalling	FXR/RXR Activation
Coagulation System	Actin Cytoskeleton Signalling	Coagulation System
Extrinsic Prothrombin Activation	Clathrin-mediated Endocytosis Signalling	Clathrin-mediated Endocytosis Signalling
Intrinsic Prothrombin Activation	Intrinsic Prothrombin Activation	Extrinsic Prothrombin Activation
EIF2 Signalling	Caveolar-mediated Endocytosis Signalling	Complement System
Complement System	Complement System	Protein Ubiquitination
Clathrin-mediated Endocytosis Signalling	Coagulation System	Intrinsic Prothrombin Activation
Protein Ubiquitination	IL-12 Signalling and Production in Macrophages	EIF2 Signalling
Regulation of eIF4 and p7056K Signalling	Extrinsic Prothrombin Activation	Remodelling of epithelial Adherens Junctions
mTOR Signalling	Atheroschlerosis Signalling	IL-12 Signalling and Production in Macrophages
Remodelling of epithelial Adherens Junctions	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
Atheroschlerosis Signalling	Cell Cycle:G2/M DNA Damage Checkpoint Regulation	Atheroschlerosis Signalling
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	14-3-3-mediated Signalling	Actin Cytoskeleton Signalling

Table 2.3. Top 15 canonical pathways detected for Set 1 analyses.

For each analysis carried out (All-C vs. All-HF, C-No vs. C-Bl, HF-No vs. HF-Bl), the top 15 canonical pathways associated with the differentially expressed proteins, detected by IPA are listed. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst; LXR = liver X receptor; RXR = retinoid X receptor; FXR = farnesoid X receptor.

All-C vs. All-HF	C-No vs. C-Bl	HF-No vs. HF-Bl
Protein Ubiquitination Pathway	Acute Phase Response Signalling	Acute Phase Response Signalling
Complement System	LXR/RXR Activation	Coagulation System
Acute Phase Response Signalling	FXR/RXR Activation	LXR/RXR Activation
Coagulation System	Coagulation System	Extrinsic Prothrombin Activation Pathway
Extrinsic Prothrombin Activation Pathway	EIF2 Signalling	Intrinsic Prothrombin Activation Pathway
5-aminoimidazole Ribonucleotide Biosynthesis I	Complement System	FXR/RXR Activation
FXR/RXR Activation	Clathrin-mediated Endocytosis Signalling	Clathrin-mediated Endocytosis Signalling
Clathrin-mediated Endocytosis Signalling	Atheroschlerosis Signalling	Protein Ubiquitination Pathway
IL-22 Signalling	Integrin Signalling	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
Role of Jak family kinases in IL-6-type Cytokine Signalling	Intrinsic Prothrombin Activation Pathway	Complement System
Intrinsic Prothrombin Activation Pathway	IL-12 Signalling and Production in Macrophages	Atheroschlerosis Signalling
Growth Hormone Signalling	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	IL-12 Signalling and Production in Macrophages
IL-9 Signalling	Extrinsic Prothrombin Activation Pathway	EIF2 Signalling
Oncostatin M Signalling	Regulation of eIF4 and p7056K Signalling	ILK Signalling
IL-3 Signalling	Regulation of Cellular Mechanics by Calpain Protease	RAN Signalling

Table 2.4. Top 15 canonical pathways detected from Set 2 analyses.

For each analysis carried out (All-C vs. All-HF, C-No vs. C-Bl, HF-No vs. HF-Bl), the top 15 canonical pathways associated with the differentially expressed proteins, detected by IPA are listed. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst; LXR = liver X receptor; RXR = retinoid X receptor; FXR = farnesoid X receptor.

Protein Name	All-C vs. All-HF		C-No vs. C-Bl		HF-No vs. HF-Bl	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
α-2-HS-glycoprotein	-	Down	Down	Up	x	-
Albumin	Down	-		-	x	-
α-1-microglobulin	Up	-	x	Up	Up	-
Apolipoprotein A1		-	Down		Down	Up
Apolipoprotein A2		-	x		Up	-
Apolipoprotein H	-	-	x		x	Up
Complement component 3	Down	-	Down		-	
Complement component 1	-	Down	x		x	-
Complement component 5	Down	-	x		Up	-
Complement component 4B		-	-		Down	Up
Complement component 9	-	-	x	-	Up	-
Complement component 4 BP α	Up	Up	Up	Up		Up
Complement component 4 BP β	x		x		x	
Complement factor B	x	-	-		Up	-
Ceruloplasmin	Down	-	Down	-		Up
C-reactive protein	Up	Up	x	Up	Down	-
Coagulation factor II (thrombin)		Down	Down			Up
Fibrinogen α chain	x	-		-		
Fibrinogen β chain	Up	-		Up	Up	
Fibrinogen γ chain		-	-		Down	
Fibronectin 1	Down	-	Down			
Ferritin, light polypeptide	Up	-	x	Up	Up	-
Heterogeneous nuclear ribonucleoprotein K	-	-	x		Down	Up
Haptoglobin	Down	Down	x		Up	-
Hemopexin		-	Up		x	-
Histidine-rich glycoprotein	-	-	x		Down	Up

Table 2.5. Continued next page.

IATI heavy chain 3	Down	Down	x	-	x	-
IATI heavy chain 4		-	x	Up	-	Up
Kallikrein B, plasma 1	-	-	-		Up	-
Lipopolysaccharide BP	Down	-	x		x	Up
MAPK 2	x	-	x		x	-
MAPK 1	x	-	x		x	x
Orosomucoid 1	x	x	x	x	Up	x
Plasminogen	-	-	-	Up		Up
Rel-A	x	x	x		x	
Serum amyloid A4, constitutive	x	x	x	-	x	Down
Serpin clade A, member 1	Down	-	x	-	Up	-
Serpin clade A, member 3		-	Up	Up		Up
Serpin clade D, member 1	-	-	x			
Serpin clade F, member 1	Up	-	-		x	
Serpin clade F, member 2	x	-	x		x	x
Stat3	x	Up	x		x	Up
Transferrin	Down	-	-	-	Up	
Transthyretin	-	-	x	-	Down	-
Von Willebrand factor	x	-	x	Up	x	-

Table 2.5. Acute phase response signalling proteins and their expression in the datasets.

The table lists all the proteins involved in Acute Phase Response Signalling and their expression in each analysis. Proteins were either upregulated (pink), downregulated (blue), not differentially expressed (-, grey) or not detected (x, white). Proteins highlighted in bold were differentially expressed in at least three analyses. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibrefed animals with blastocyst; BP = binding protein; IATI = inter-α-trypsin inhibitor; MAPK = Mitogen-activated protein kinase; Rela = V-rel avian reticuloendotheliosis viral oncogene homolog A; Serpin = serpin peptidase inhibitor; Stat3 = Signal transducer and activator of transcription 3.

Protein Name	All-C vs. All-HF		C-No vs. C-Bl		HF-No vs. HF-Bl	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 1
Coagulation Factor II	Up	Down	Down	Up	Down	Up
Coagulation Factor V	x	-	Up	x	x	x
Coagulation Factor VII	x	Down	x	x	x	x
Coagulation Factor IX	Up		Down	Up	-	Up
Coagulation Factor X		-	x	-	Up	
Coagulation Factor XII	-	-	x	Up	Down	
Coagulation Factor XIII	Up	-	x		x	
Fibrinogen α-chain	-	-	Down	-	Down	
Fibrinogen β-chain	Up	-		Up	Up	
Fibrinogen γ-chain		-	x		Down	
Kallikrein C	-	-	Down	Up	Up	-
Plasminogen	-	-	x			Up
Protein S	x	x	x		Down	
Serpin, clade A, member 1	Down	-	x	-	Up	-
Serpin, clade A, member 5		-	x	-		Up
Serpin, clade D, member 1	-	-	x	-		
Serpin, clade F, member 2	x	-	x	Up	x	x
Protein C	Up	Down	x		Down	Up
Von Willebrand Factor	x	-	x		x	-

Table 2.6. Coagulation system proteins and their expression in the datasets.

The table lists all the proteins involved in Coagulation System and their expression in each analysis. Proteins were either upregulated (pink), downregulated (blue), not differentially expressed (-, grey) or not detected (x, white). Proteins highlighted in bold were differentially expressed in at least three analyses. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst; Serpin = serpin peptidase inhibitor.

Protein Name	All-C vs. All-HF		C-No vs. C-Bl		HF-No vs. HF-Bl	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 1
Albumin	Down	-	Down	-	x	-
α-1-B glycoprotein	x	-	x	Up	x	x
α-1-microglobulin	Up	-	x		Up	-
α-2-HS-glycoprotein	-	-	Down		x	-
Apolipoprotein A1	Up	Down			Down	Up
Apolipoprotein A2		-	x		Up	-
Apolipoprotein A4		-	Down			Up
Apolipoprotein B	x	-	x			-
Apolipoprotein C2	Up	-	x			-
Apolipoprotein C3		-	Down	Down	Down	Down
Apolipoprotein D	Down	-		Up		Up
Apolipoprotein E	Up	-	Up		Up	
Apolipoprotein F	x	-	x	x	x	
Apolipoprotein H	-	-	x	Up	x	
Apolipoprotein M	x	Down	Down		Up	Down
Complement component 3	Down	-			-	Up
Complement component 9	-	-	x	-	Up	-
Complement component 4B	Down	-	-	Up	Down	Up
Clusterin	Up	-	Down			
Fatty acid synthase	x	-	Up			-
Fetuin B	x	Up	x		x	-
Fibrinogen α chain	-	-	Down	-	Down	Up
Forkhead box O1	x	Up	x	x		x
Hemopexin	Down	-	Up	Up	x	-
IATI heavy chain, member 4		-	x	Up	-	Up
Kininogen 1	-	-	Down	-	-	-
Lecithin-cholesterol AT	-	-	x	Up	x	-
Orosomucoid 1	x	x	x	x	Up	x

Table 2.7. Continued next page.

Paraoxonase 1	Down	-	x	-	Down	-
Serum amyloid A4	x	x	x	-	x	Down
Serpin, clade A, member 1	Down	-	x	-	Up	-
Serpin, clade F, member 1	Up	-	-	Up	x	Up
Serpin, clade F, member 2	x	-	x	Up	x	x
Transferrin	Down	-	-	-	Up	Up
Transthyretin	-	-	x	-	Down	-
Vitamin D binding protein	Up	-	x	Up		-

Table 2.7. FXR/RXR activation proteins and their expression in the datasets.

The table lists all the proteins involved in FXR/RXR activation and their expression in each analysis. Proteins were either upregulated (pink), downregulated (blue), not differentially expressed (-, grey) or not detected (x, white). Proteins highlighted in bold were differentially expressed in at least three analyses. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst; AT = acyltransferase; IATI = inter-α-trypsin inhibitor; Serpin = serpin peptidase inhibitor.

Protein Name	All-C vs. All-HF		C-No vs. C-Bl		HF-No vs. HF-Bl	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 1
α-1-B glycoprotein	x	-	x	Up	x	x
α-2-HS glycoprotein	-		Down		x	-
Albumin	Down	-		-	x	-
α-1-Microglobulin	Up	-	x	Up	Up	-
Apolipoprotein A1		-	Down		Down	Up
Apolipoprotein A2		-	x		Up	-
Apolipoprotein A4		-	Down			Up
Apolipoprotein B	x	-	x			-
Apolipoprotein C2	-	-	x		-	-
Apolipoprotein C3	Up	-	Down	Down	Down	Down
Apolipoprotein D	Down	-		Up		Up
Apolipoprotein E	Up	-	Up		Up	
Apolipoprotein F	x	-	x	x	x	
Apolipoprotein H	-	-	x	Up	x	
Apolipoprotein M	x	-	Down		Up	Down
Complement component 3	Down	-			x	Up
Complement component 9	-	-	x	-	Up	-
Complement component 4B	Down	-	-	Up	Down	Up
CD14 molecule	-	-	Down			Up
Clusterin	Up	-				Up
Fatty acid synthase	x	-	Up	-		-
Fibrinogen α chain	-	-	Down	-		Up
Vitamin D binding protein	Up	-	x	Up		-
Hemopexin	Down	-	Up		x	-
IATI heavy chain 4		-	x		-	Up
Kininogen 1	-	-	Down	-	-	-
Lipopolysaccharide BP	Down	-	x	Up	x	Up
Lectin-cholesterol AT	-	-	x		x	-
Lysozyme	x	Down	x		Up	-
Orosomucoid 1	x	x	x	x		x
Paraoxonase 1	Down	-	x	Up	Down	-

Table 2.8. Continued next page.

Rel-A	X	-	x		x	x
Serum amyloid A4	x	x	x	-	x	Up
Serpin, clade A, member 1	Down	-	x	-	Up	-
Serpin, clade F, member 1	Up	-	-	Up	x	Up
Serpin, clade F, member 2	x	-	x		x	x
Transferrin	Down	-	-	-	Up	Up
Transthyretin	-	-	x	-		-

Table 2.8. LXR/RXR activation proteins and their expression in the datasets.

The table lists all the proteins involved in LXR/RXR activation and their expression in each analysis. Proteins were either upregulated (pink), downregulated (blue), not differentially expressed (-, grey) or not detected (x, white). Proteins highlighted in bold were differentially expressed in at least three analyses. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst; AT = acyltransferase; IATI = inter-α-trypsin inhibitor; Rela = V-rel avian reticuloendotheliosis viral oncogene homolog A; Serpin = serpin peptidase inhibitor.

Analysis	Ratio	Mascot Score	Name
Set 1 All-C vs. All-HF	0.33	5718.5	Fibronectin
	0.34	2136.9	Heat shock 90 kDa protein 1
	0.35	1767.9	**Ceruloplasmin**
	0.38	2572.8	Heat shock protein HSP 90-α
	0.41	6645.4	**Complement component C3**
	1.57	5326.7	**Vitamin D binding protein**
	1.6	1805.4	**Complement component C4 BP α**
	1.77	4924.3	**Apolipoprotein A4**
	1.79	2528.5	Tropomyosin β chain isoform 2
	1.8	4016.3	**Complement factor H**
	1.82	2228.7	Desmin
	2.2	2020.5	**Coagulation factor IX**
	2.32	1987.9	**Coagulation factor II**
Set 2 All-C vs. All-HF	1.33	2669.1	Endoplasmin
	1.36	2209	Inter-α-trypsin inhibitor heavy chain H2
	1.47	2920.2	**Coagulation Factor II**
	1.51	1698.7	Inter-α-trypsin inhibitor heavy chain H1
	1.54	1656.3	Inter-α-trypsin inhibitor heavy chain H1

Table 2.9. Continued next page.

Set 1 C-No vs. C-Bl	1.61	4599.9	**Serum albumin**
	1.82	4820.7	**Apolipoprotein A1**
Set 2 C-No vs. C-Bl	1.97	2516.7	**Apolipoprotein E**
	2.31	2108.2	Desmin
	0.72	1531.7	Programmed cell death protein 4
	0.74	2104.4	Heterogeneous nuclear ribonucleoprotein L-A
Set 1 HF-No vs. HF-Bl	0.25	1875.8	Transferrin
	0.33	1542.4	Inter-α-trypsin inhibitor heavy chain H1
	2.9	6997.5	**Apolipoprotein A1**
	4.62	1658.3	Heterogeneous nuclear ribonucleoprotein K
	6.72	4974.9	Vimentin
Set 2 HF-No vs. HF-Bl	1.72	2301.4	Serpin peptidase inhibitor, clade C, member 1
	1.73	1614.7	IgA heavy chain
	1.79	3977.8	**Plasminogen**
	1.79	5229.8	**Complement component C3**
	2.49	2806.2	**Coagulation Factor II**
	3.27	1524.8	Vitamin K-dependent protein C
	0.4	1561.7	Calmodulin
	0.49	3379.9	Tropomyosin α-1 chain
	0.52	3691.5	Tropomyosin α-3 chain
	0.56	3704.6	Tropomyosin 3
	0.59	3254.3	Elongation factor 1-α
	0.61	4871.6	Tropomyosin β chain isoform 2

Table 2.9. List of proteins in each analysis with the highest and lowest heavy/light ratios and highest mascot scores.

The proteins listed have heavy/light ratios higher than 1.50 or lower than 0.67, apart from Set 2 All-C vs. All-HF which have ratios higher than 1.30 or lower than 0.77. Additionally, the proteins also have mascot scores of higher than 1500, therefore narrowing the list.

Candidate	Rationale for selection
Involved in FXR/RXR and LXR/RXR activation pathways	
Apolipoprotein A4	High ratio (1.77) in Set 1 All-C vs. All HF Upregulated in four analyses Downregulated in one analysis
Apolipoprotein E	High ratio (1.97) in Set 2 C-No vs. C-Bl Upregulated in five analyses Possible upstream regulator
Apolipoprotein M	Upregulated in two analyses Downregulated in three analyses
Involved in acute phase response and coagulation pathways	
Ceruloplasmin	Low ratio (0.35) in Set 1 All-C vs. All-HF Upregulated in two analyses Downregulated in two analyses
Complement component 4 binding protein α	High ratio (1.60) in Set 2 All-C vs. All-HF Upregulated in two analyses Downregulated in two analyses
Clusterin	Upregulated in three analyses Downregulated in two analyses
Inter-α-trypsin inhibitor heavy chain H1	High ratio (1.51) in Set 2 All-C vs. All-HF Low ratio (0.33) in Set 1 HF-No vs. HF-Bl
Fibrinogen β	Upregulated in four analyses Downregulated in one analysis
Lipopolysaccharide binding protein	Upregulated in two analyses Downregulated in one analysis
Plasminogen	High ratio (1.79) in Set 2 HF-No vs. HF-Bl Upregulated in three analyses
Tropomyosin 1	Low ratio (0.61) in Set 2 HF-No vs. HFBl Upregulated in four analyses

Table 2.10. List of candidates for validation.

The candidates for validation are involved in either FXR/RXR and LXR/RXR activation or acute phase response and coagulation. Candidates can either have very high or very low heavy/light ratios in certain analyses and/or upregulated or downregulated in multiple analyses.

2.4. Discussion

2.4.1. The digestion-depletion method was not a reliable method for selectively depleting abundant proteins compared to the Proteominer kit

The digestion-depletion method developed by Fonslow *et al.*, (2013) involves the addition of a very small amount of a protease, such as trypsin. According to Michaelis Menton kinetics, the rate of hydrolysis of a substrate is proportional to its starting concentration (Johnson and Goody, 2011). With this principle, the protease should selectively digest proteins of high abundance in the sample. Fonslow *et al.*, (2013) suggested the addition of protease in a ratio of 1: 25,000 protein: protease. When carried out on pFF, only after the addition of 360 ng of trypsin per mg protein, was the sample thoroughly digested. Fonslow *et al.*, (2013) depleted yeast proteins which are less dynamic than pFF, so it is plausible that pFF would require more trypsin. However, it was not known which specific proteins were digested and removed. The current study suggests that the larger proteins were digested, resulting in more low molecular weight proteins. Ye *et al.*, (2014) carried out a proteomic study to test the robustness of the digestion-depletion method as a means of removing high abundance proteins. A total cell lysate was digested with trypsin at a protein-protease mass ratio of 25,000:1 for 12 hours as with Fonslow *et al.*, (2013), half of which was further digested overnight with the addition of trypsin at a protein-protease ratio of 50:1 (Ye *et al.*, 2014). Following LC-MS/MS, no notable difference in the distribution between samples digested for 12 hour and sample digested overnight, suggesting that digestion was likely to be independent of abundance. It has been suggested that varying digestion times in combination with the use of other proteases with different site specificity constants and different molecular weight-cut off filter sizes to suit the sample proteome would provide more efficient depletion (Fonslow *et al.*, 2013). To do this would be time consuming and it was more productive to test an alternative, more promising depletion method.

The efficiency of the Proteominer enrichment kit has been demonstrated in various other types of samples and has proven to be both efficient and reproducible in enriching plant proteins, human serum, human plasma, human follicular fluid (hFF),

bovine serum, porcine serum, equine FF and most importantly pFF (Zolotarjova *et al.*, 2005; Dwivedi *et al.*, 2010; Marco-Ramell and Bassols, 2010; Fahiminiya *et al.*, 2011a; Di Girolamo *et al.*, 2013). The study in which the kit was successfully used on pFF was most encouraging (Fahiminiya *et al.*, 2011a). In that study, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) showed that the Proteominer kit removed a great deal of abundant proteins such as albumin, transferrin, haptoglobin, apolipoprotein A1 and light and heavy chains of immunoglobulin gamma from pFF. Therefore, there was greater confidence in using the Proteominer as a means of essential reduction in dynamic range.

The efficient depletion of abundant proteins led to the detection of more proteins in each analysis than reported in previous pFF studies (Bijttebier *et al.*, 2009; Sun *et al.*, 2011). The reason for the greater number of proteins detected is believed to be attributed to the use of the Proteominer kit to enrich proteins of lower abundance. For example, Sun *et al.*, (2011) identified 300 protein spots on the 2-D PAGE gels, following depletion with the ProteoExtract Albumin/IgG removal kit, whilst Bijttebier *et al.*, (2009) identified 69 proteins without depletion of abundant proteins. This observation was reinforced with the results of proteomic studies in other species. Fu *et al.*, (2016) identified 363 proteins in buffalo FF when depletion had not been carried out and Maniwa *et al.*, (2005) identified 40 spots following depletion with the Aurum serum protein mini kit.

Studies carried out by (Fahiminiya *et al.*, 2011a) have compared different depletion methods on FF of various species including pigs. They observed that more abundant proteins in pFF were removed by the ProteomeLab IgY-HAS than the Multi Affinity Removal System-6 (MARS-6) kit but both depletion methods were more effective on canine FF and hFF compared to pFF samples (Fahiminiya *et al.*, 2012). However, when using the Proteominer enrichment kit, the depletion efficiencies were more comparable between hFF, pFF and equine FF with depletion efficiencies of 98.71%, 98.49% and 98.52% respectively (Fahiminiya *et al.*, 2011a). Apart from the work of Fahiminiya *et al.*, (2011a, b, 2012) there are very few efficient proteomic analyses with animal FF samples especially studies using a similar workflow as the one used in this

study. Most of these studies have utilised 2D gel separation and protein spot excision for analysis, thereby eliminating the detection of a large number of proteins.

Most of the proteomic studies published in the last five years which used similar workflows to the one used in this study have analysed hFF and have utilised the MARS-14 kit which removes the 14 most abundant proteins in hFF. The use of immuno-specific depletion kits has enabled the detection of approximately 750 proteins in hFF (Ambekar *et al.*, 2015; Zamah *et al.*, 2015). However, earlier studies using this technology have acquired fewer detected proteins (between 75 and 480, Kushnir *et al.*, 2012; Ambekar *et al.*, 2013). Other methods have included the addition of ice cold ethanol and the ProteoSeek Albumin/IgG removal kit which allowed for the detection of 227 and 89 proteins respectively (Severino *et al.*, 2013; Twigt *et al.*, 2015). Interestingly, Regiani *et al.*, (2015) detected 535 proteins with no depletion method. This implies that other factors in preparation also play a role in efficiency, such as technical handling, isotopic labelling and sensitivity of the mass spectrometer. This can easily be applied to the results of this study, as the results that will be discussed below will postulate possible variations in the preparation of Set 1 and Set 2 samples.

2.4.2. The Set 2 dataset detected twice as many proteins and appears to be more reliable compared to the Set 1 dataset

Many published studies present just one replicate of the proteomic analyses. In this study, each proteomic analysis was carried out twice to provide additional confidence in the results obtained, producing Set 1 and Set 2 datasets for each analysis. The original aim was to combine Set 1 and Set 2 results, using only the data that was in agreement between the two datasets in subsequent analyses. However, there were nearly double the number of proteins detected in Set 2 compared to Set 1. This made it difficult to combine the two datasets and it was unclear which set of results to have more confidence in. After further analysis of the data, the difference between Set 1 and Set 2 datasets became more apparent.

The differences between Set 1 and Set 2 datasets persisted when identifying differentially expressed proteins. The detection of differentially expressed proteins between control and high fibre pFF is a promising indication that nutrition alters the composition of pFF. However, the Set 2 datasets have a more normal distribution, making it easier to identify outliers of the cut-off points. However, this was not the case as only the expression of 100–150 proteins were in agreement between Set 1 and Set 2 datasets, whilst 25–75 proteins have contradicting expressions; upregulated in one set and downregulated in the other. Most of proteins detected were differentially expressed in one set (usually Set 1) and unchanged in the other (usually Set 2).

The differences observed are likely due to technical improvements from carrying out the workflow the second-time round. All these features suggest greater confidence in Set 2 dataset, although this cannot be confirmed. Therefore, instead of combining the results of Set 1 and Set 2 datasets, or disregarding Set 1 dataset altogether, subsequent bioinformatic analyses were carried out separately on each dataset.

2.4.3. Canonical pathways previously implicated in ovarian function are associated with the differentially expressed proteins in this study

As discussed in the chapter 1, sections 1.4.3, 1.4.5 and 1.4.6, there have been several published studies that have used proteomic techniques as a means to generate logical hypotheses or to identify potential biomarkers for perturbed ovarian function and/or reproductive success. The methods used in this particular study have been heavily influenced by the previous published studies, in particular regarding the workflows. This study also used the proteomic data as a hypothesis generating approach as opposed to identifying known targets of reproductive function. This logical, survey approach was carried out with the aim of discovering novel molecules and/or pathways that could be implicated. The challenging aspect of using bioinformatic tools such as DAVID or IPA was choosing an avenue for further analysis as there were several directions in which to take the study, including potential upstream regulators and network analyses. Using the top canonical pathways gave direct associations with the differentially expressed proteins, making it easier to identify candidates for validation. Additionally, the use of information from the proteomic data such as large heavy/light

ratios and mascot scores during candidate selection give numerical source of confidence in the choice of proteins for validation. With this in mind, IPA analysis was used to identify the biological relevance of the dataset and the results made sense in terms of biological context and became useful in generating potential hypotheses.

Despite the differences in Set 1 and Set 2 datasets, similar outputs were obtained when analysed with IPA software. These outputs included the association of the differentially expressed proteins with inflammatory pathways. This was encouraging as the inflammatory processes were heavily involved in ovarian function and have been implicated in other follicular fluid (FF) proteomic analyses (Angelucci *et al.*, 2006; Hanrieder *et al.*, 2008, 2009; Jarkovska *et al.*, 2010; Twigt *et al.*, 2012; Ambekar *et al.*, 2013; Bianchi *et al.*, 2013; Shen *et al.*, 2017). Espey, (1980, 1994) was one of the first to comprehensively describe ovulation as an inflammatory process, by weakening the follicular wall. More recently, this hypothesis is now more established, as the processes governing ovulation are better understood. Prior to ovulation, the LH surge increases the concentrations of prostaglandins such as oestradiol, which enhance inflammation by releasing proteolytic enzymes to degrade the connective tissue of a follicle, preparing it for rupture at ovulation (Boots and Jungheim, 2015). Several cell types and molecules of the immune system such as leukocytes (particularly eosinophils in pigs) and cytokines are recruited to the ovarian follicle to aid in the inflammatory response to the rupture event of ovulation (Standaert *et al.*, 1991). Following this rupture event, extensive tissue remodelling is required for the formation of the *corpus luteum* and subsequently *corpus albicans*. In every step of this inflammatory process, proteins such as complement components, coagulation factors and apolipoproteins, all of which were detected in this study, play a role. For example, plasminogen, which is involved in the coagulation system and acute phase response signalling, is a precursor to plasmin, a proteolytic factor involved in the weakening the follicular wall prior to ovulation (Mosesson, 2005). Additionally, fibrinogen, which is also involved in the coagulation system and acute phase response signalling, is a precursor to fibrin, a protein that polymerises with blood platelets during blood clotting (Cesarman-Maus and Hajjar, 2005).

Other canonical pathways associated with the differentially expressed proteins in this study were LXR/RXR and FXR/RXR pathways. The general function of LXR and FXR transcription factors is to produce proteins involved in lipid, cholesterol, glucose, and triglyceride homeostasis (Kalaany and Mangelsdorf, 2006; Wang *et al.*, 2008; Zhao and Dahlman-Wright, 2010). Their roles in reproduction are not well recognised although there are studies that give an indication of their importance. For example, treating lutenised human granulosa cells with LXR agonists resulted in a 50% reduction of progesterone production by those cells, which is unsurprising as steroidogenesis is dependent on the supply of cholesterol by lipoproteins produced by LXR/RXR activation (Drouineaud *et al.*, 2007). Similarly, the *corpora lutea* of ewes that had spontaneous and prostaglandin F2 α-induced luteolysis had decreased cholesterol uptake and increased LXR activity (Seto and Bogan, 2015). Most interestingly, LXR α and β deficient (knockout) mice display typical features of ovarian hyperstimulation syndrome, including increased oestradiol production as well as deregulation of inflammation (Mouzat *et al.*, 2009). This suggests that there is the possibility of a synergetic mechanism of the top canonical pathways detected in this study. It is also worth noting that circulating oestradiol concentration decreases with the high fibre diet (Ferguson *et al.*, 2007) and according to the Set 2 results presented in this study, LXR/RXR activity decreased with blastocyst formation. The role of FXR in reproduction is less clear although immunohistochemistry demonstrated that rabbit reproductive tissues (ovary, oviduct, uterus and vagina) stained positive for FXR (Anaya-Hernández *et al.*, 2014).

In addition to the repeated association of these pathways with the differentially expressed proteins in the three different analyses in this study, other studies have identified the same pathways in FF of different species. For example, the majority of proteins detected in buffalo FF are involved in the complement and coagulation cascades (Fu *et al.*, 2016). The three most significant pathways associated with the differentially expressed proteins in hFF of women undergoing controlled ovarian hyperstimulation (COH) were acute phase response signalling, LXR/RXR activation and coagulation system (Wu *et al.*, 2015). Analysis with DAVID software identified the association of hFF proteins with inflammation, regulation of inflammation, acute

phase as well as protein-lipid complex and lipid metabolism and transport (Bianchi *et al.*, 2013). IPA revealed the association of differentially expressed proteins between FF from different IVF outcomes were involved in coagulation, acute phase response signalling, complement system and LXR/RXR activation (Severino *et al.*, 2013). Kushnir *et al.*, (2012) identified a 40% higher significance score for complement system in hFF of patients who miscarried following an IVF cycle and 40% lower significance score in hFF of patients with no pregnancy following IVF. The similarity in proteomic results from FF of different species adds weight to the results in this particular study, but also gives an indication as to the importance of these pathway's biological functions.

2.4.4. The limitations of the proteomic workflow

There are always ways in which an experiment can be improved. There could have been the option of repeating the experiments a second time to have three repeats or to do pair wise comparisons with individual animals. However, these alternative methods would utilise more valuable samples. Nevertheless, the main limitation with carrying out a proteomic experiment with pFF was always overcoming its dynamic range. Although the use of the Proteominer kit resulted in efficient depletion of abundant proteins and the detection of approximately 1000 proteins, it is still not entirely known what was removed. There is the potential that using the kit results in different amounts of proteins being removed with each repeat, which would explain the varying results between Set 1 and Set 2 datasets. Therefore, this variation in results made it difficult to identify subsequent research direction without the validation of one set of the data.

2.4.5. The validation of candidates, subsequent experiments and alternative research directions

As part of the proteomic workflow, it is typical for a sub-selection of proteins to be validated by techniques such as western blotting and enzyme-linked immunosorbant assay (ELISA). Western blotting is the most utilised method of validation and has been used to confirm the increased expression of serpin family D member 1 and decreased expression of vimentin and peroxiredoxin-1 in FF of large buffalo follicles compared

to FF of small buffalo follicles (Fu *et al.*, 2016). It was also used to confirm the upregulation of α1-antitrypsin, apolipoprotein A1 and transferrin in hFF of polycystic ovary syndrome (PCOS) patients compared to normal women and the increased concentration of α1-antitrypsin in hFF of women undergoing COH (Dai and Lu, 2012; Wu *et al.*, 2015). Sun *et al.*, (2011) used western blotting to confirm the increased expression of gelsolin and apolipoprotein A1 in pFF of cystic pig follicles compared to pFF of large follicles and the increased expression of transferrin in pFF of large follicles compared to medium sized follicles. However, ELISAs are increasingly being used due to their increased sensitivity, and have been used to confirm the downregulation of heparan sulphate proteoglycan 2, ampherigulin and fibronectin and the up-regulation of α1-antitrypsin in hFF of women with PCOS (Ambekar *et al.*, 2015).

2.4.6. Conclusions

Using a proteomic workflow optimised for the detection of low abundance proteins in pFF, proteins have been identified as being differentially expressed between pFF of control-fed pigs and pFF of high fibre-fed pigs. Furthermore, within each feeding group, differentially expressed proteins were identified between pFF of pigs whose oocytes produced a blastocyst and pigs whose oocytes did not produce a blastocyst following IVF. These differentially expressed proteins were associated with canonical pathways previously implicated in ovarian function and physiology, including pathways involved in inflammation. The next step was to carry out western blots for a selection of candidates to both validate the proteomic data and focus the direction of the study.

3. Candidate Confirmation of Proteomic Analysis by Western Blotting

3.1. Introduction

In chapter 2, two proteomic analyses (Set 1 and Set 2) were carried out on pooled porcine follicular fluid (pFF) from gilts fed either a control barley-based diet (All-C) or a high fibre diet supplemented with unmolassed sugar beet pulp (All-HF). Within each dietary group, proteomic analyses were also carried out between pFF of gilts whose oocytes produced a blastocyst (C-BL and HF-Bl) following *in vitro* fertilisation (IVF) and pFF of gilts whose oocytes did not produce a blastocyst (C-No and HF-No, respectively). Several hundred differentially expressed proteins were detected between the sample pools. Ingenuity Pathway Analysis (IPA) revealed the association of these differentially expressed proteins with different canonical pathways, including the coagulation system, acute phase response, liver X receptor (LXR)/retinoid X receptor (RXR) activation and farnesoid X receptor (FXR)/RXR activation. Eleven candidate proteins associated with these canonical pathways were selected for analysis by western blotting to confirm their relative expression between the sample pools and determine whether these relative expressions in unaltered (non-depleted samples) was in agreement with the expressions detected in the proteomic analyses.

3.1.1. The candidates for validation and their general biological functions

Apolipoproteins involved in LXR/RXR and FXR/RXR activation

The candidates involved with LXR/RXR and FXR/RXR activation were apolipoprotein A4, apolipoprotein E, clusterin (also known as apolipoprotein J) and apolipoprotein M. These proteins either inhibit the expression of target genes of LXR, RXR and FXR, or are targets of these transcription factors themselves (Zhang *et al.*, 2008; Zhu *et al.*, 2011; Seo *et al.*, 2013; Ren *et al.*, 2015). These proteins have major functions in high-density and low-density lipoprotein formation, and therefore are

involved in the transport and homeostasis of lipids such as cholesterol (Jenne *et al.*, 1991; Gelissen *et al.*, 1998). A change in the apolipoproteins could be indicative of a change in cholesterol, and therefore subsequent steroidogenesis or a change in oocyte energy consumption.

Acute phase response proteins involved in immune response and inflammation

The process of ovulation is often thought of as an inflammatory process, involving cytokines and acute phase response systems (Suchanek *et al.*, 1990; Büscher *et al.*, 1999), and metabolic perturbations can alter the concentration of the associated proteins (Sessions-Bresnahan and Carnevale, 2014). The proteomic analyses identified the differential expression of many acute phase response proteins between the pFF pools analysed, including ceruloplasmin, complement component 4 binding protein α, plasminogen, fibrinogen β and lipopolysaccharide binding protein. Acute phase proteins are defined as hepatic proteins with concentrations that increase or decrease in serum by at least 25% in the first 7 days after tissue damage (Kushner, 1982). Ovulation involves the rupturing of the ovarian follicular wall and an associated damage to ovarian tissue. Therefore, the flux of acute phase proteins prior to, during and after ovulation is to be expected.

The acute phase proteins can be subdivided into further groups such as coagulation proteins (plasminogen and fibrinogen), transport proteins (ceruloplasmin), complement components and protease inhibitors (Van Leeuwen and Van Rijswijk, 1994). Therefore, the acute phase response, coagulation system and complement system can be considered to act concurrently, with the accumulation of acute phase proteins and complement proteins to prepare for ovulation, whilst coagulation proteins prepare for tissue re-structure after ovulation.

Plasminogen and fibrinogen are also involved in the coagulation system, but with opposing functions. Fibrinogen β forms the full structure of fibrinogen which is involved the formation of blood clots whilst plasminogen is the inactive precursor to plasmin, which carries out fibrinolysis, the process by which blood clots are broken down (Cesarman-Maus and Hajjar, 2005; Mosesson, 2005). The fibrinolysis process

extends to the ovary as plasmin has been shown to be directly involved in rupturing of the follicular wall prior to ovulation, by acting as a proteolytic enzyme (Beers, 1975; Ogiwara *et al.*, 2012). Additionally, it has been suggested that fibrinogen is involved in the blood clotting process post-ovulation (Parrott *et al.*, 1993). Therefore, the differential expression of plasminogen and fibrinogen may be in synergy.

The other short-listed candidate proteins associated with acute phase response have a variety of functions. Alterations in serum concentrations of cations such as copper and iron occur during the acute phase response and the copper-binding protein ceruoplasmin is directly involved in the circulating levels of these cations in pigs (Lee *et al.*, 1968; Kushner, 1982). It has also been suggested that ceruloplasmin possesses scavenging properties, targeting oxygen-derived free radicals during inflammation (Goldstein *et al.*, 1982). Lipopolysaccharide binding protein is involved in the innate immune response to bacterial infection and the activation of nuclear factor-κβ and mitogen–activated protein kinases to produce inflammatory proteins (Han *et al.*, 1994; Hatada *et al.*, 2000; Ding and Jin, 2014). Complement component 4 binding protein is the main regulatory protein of the complement system and forms a complex with Protein S to modulate the anticoagulant properties of activated protein C (Rezende *et al.*, 2004).

Inter-α-trypsin inhibitor heavy chain 1 and tropomyosin 1

Inter-α-trypsin inhibitor 1 and tropomyosin 1 were not associated with the top canonical pathways identified by IPA analysis. However, inter-α-trypsin inhibitor 1 was shown to be one of the most differentially expressed proteins in Set 1 HF-No versus HF-Bl analysis and Set 2 All-C versus All-HF analysis whilst tropomyosin 1 was one of the most differentially expressed proteins in Set 2 HF-No versus HF-Bl. Additionally, they have interesting biological functions within the ovary. During cumulus expansion, cumulus-oocyte complexes synthesise hyaluronan which associates with proteins and proteoglycans to form an expanded extracellular matrix, where the heavy chains of serum derived inter-α-trypsin inhibitor covalently binds to hyaluronan and contribute to the matrix structure (Nagyova *et al.*, 2004). Tropomyosin polypeptides have been identified in mouse preimplantation conceptuses, forming a

major portion of the heat-stable cytoskeletal protein fraction of blastomeres (Clayton and Johnson, 1998).

3.1.2. Hypothesis and experimental aims

Following the proteomic analyses, I hypothesised that the expression of the selected protein candidates would be different between the pooled pFF samples, and that these differences could be demonstrated by western blotting. The aim of this work was to confirm the relative expression of the selected candidate proteins in the pFF samples to validate the proteomic analyses carried out in chapter 2. The candidates have relevant biological functions and the confirmation of their differential expression would indicate the potential processes involved in high fibre diet-induced reproductive benefits.

3.2. Materials and Methods

3.2.1. Sample preparation

The pFF pools were prepared as described in chapter 2, section 2.2.2; these pools being the All-C, All-HF, C-No, C-Bl, HF-No and HF-Bl pFF pools. Each pool was then diluted by a factor of 20 with deionised water (dH_2O). Additionally, porcine plasma from a pregnant gilt (nine months old) at gestational day 60 was collected and diluted by a factor of 20. Plasma from the gilts that provided the pFF was not available. Allantoic fluid associated with a day 60 foetus from a different (nine-month-old) gilt was also collected. The plasma would serve as a positive reference whilst the allantoic fluid would serve as a negative control. The expression values of each protein in the pFF samples could then be normalised by the expression in the plasma. The concentration of the diluted pFF pools, diluted porcine plasma and porcine allantoic fluid were determined by protein assays as described in chapter 2, section 2.2.3. The samples were stored at -20°C, until required.

3.2.2. Optimisation of western blot procedure

A series of optimisation procedures were carried out, resulting in the protocol described in this chapter. Firstly, the decision was made to use total protein quantification instead of using an internal standard (β-actin) as described in Eaton *et al.*, (2013). This was because there were very few options for internal standards that would be suitable for serum- and plasma-type samples and there was a concern that these proteins could be differentially expressed between the pooled samples. Secondly, for each protein candidate, antibody assays were carried out to determine the optimum concentration to use for each antibody as well as sample assays to identify the optimum protein loading quantity for each protein candidate. Thirdly, the iBlot 2 system was selected as a method for transferring proteins from the gel to the membrane as this was not only a time-efficient method but also because the commercially available, preprepared materials (such as the transfer stacks) would reduce the potential variability between technical repeats.

3.2.3. One-dimensional polyacrylamide gel electrophoresis for western blot

All reagents and materials were supplied by Life Technologies unless specified. Samples (pFF, plasma and allantoic fluid) were prepared in microtubes which contained 2.5x protein required for each gel and were made to the same volume with dH_2O. They also contained 35% sodium dodecyl sulphate (SDS) working solution, composed of 2.5:1 (v:v) 4X SDS sample buffer:10X reducing agent. A blank well solution was made to fill the intermediate wells which did not contain samples (35% SDS working solution and 65% dH_2O). Samples and blanks were heated to 90 ºC for 10 minutes. The samples and blanks were split into individual wells of two 17-well NuPAGE Novex Tris-Acetate gels so that wells contained either 16, 8 or 6 µg of pFF, plasma or allantoic fluid, depending on which candidate was targeted; 8 µg for apolipoprotein M, 6 µg for lipopolysaccharide binding protein and 16 µg for the remaining candidates. Precision Plus Protein Dual Xtra Standards (Biorad) was used as the ladder. Therefore, the well layout from left to right was ladder, plasma, All-C pFF, All-HF pFF, C-No pFF, C-Bl-pFF, HF-No pFF, HF-Bl pFF and allantoic fluid, with a black well in between and on the first and last wells. Electrophoresis was carried out between 100–200 V for 50–200 minutes in an X-Cell Surelock Mini Cell with running buffer composed of 950 mL dH_2O and 50 mL NuPAGE MOPS Running Buffer, until the samples ran through to the bottom of the gel. Each candidate repeat had two gels which were run in the same tank at the same time as one gel was for transfer, the other for staining. Gels that were stained were incubated with 20 mL of Instant Blue Protein Stain (Expedeon, 25 Norman Way, Over Cambridgeshire, CB24 5QE, UK) for 1 hour, washed with 50 mL of dH_2O for 2 x 30 minutes with stirring and then incubated overnight with 50 mL of dH_2O and 10 mL 20% sodium chloride (Fisher Scientific) with stirring until scanning.

3.2.4. Western blot transfer with iBlot 2

All reagents and materials were supplied by Thermo Fisher Scientific unless specified. The iBlot transfer polyvinylidene fluoride (PVDF) stacks were inserted onto the iBlot machine and soaked with the running buffer. Gels were released from the cassette and transferred onto the top of the transfer stacks on the PVDF membranes and filter paper

(pre-soaked in dH_2O) was layered on top of the gels. The machine was ran for 7 minutes at 20 volts. After transfer, the tops of the stacks were removed, and the membranes were trimmed to the size of the gels and the top right corners of the membranes were cut off above the protein ladder. Membranes were washed in 10 mL tris buffered saline with Tween 20 (TBST) for 2–5 minutes and blocked in Odyssey Blocking Buffer (Licor Bioscience, Cambridge, UK) with 0.1% Tween-20 (Amresco, 28600 Founain Parkway Solon, Ohio, 44139, USA) at room temperature on a roller for 1 hour.

3.2.5. Incubation of membranes in antibodies

Membranes were incubated in a primary antibody solution overnight at 4 °C on a roller. The primary antibody solutions were composed of different dilutions of primary antibodies (**Table 3.1**) in blocking buffer with 0.1% Tween-20. Primary antibodies were selected if they were stated to be specific or predicted to work with porcine samples. In the morning, the primary antibody solutions were discarded and the membranes were washed four times with 30 mL of 1X TBST for 5 minutes per wash on the rocker, turning the membrane over between washes. Membranes were then incubated in a secondary antibody solution (again in blocking buffer with 0.1% Tween-20) for 1 hour on the rocker in the dark. Different secondary antibodies were used (**Table 3.1**) depending on the primary antibody. The secondary antibody solution was discarded and the membranes were washed six times with 30 mL of 1X TBST for 5 minutes per wash on the rocker, turning the membrane over between washes. Membranes were then imaged using the Odyssey Licor scanner (Licor Biosciences), along with their corresponding gels. Of the original 11 candidates, working antibodies were found for apolipoprotein A4, apolipoprotein M, ceruloplasmin, clusterin, lipopolysaccharide binding protein, plasminogen and fibrinogen β. Therefore, the relative expression of these proteins could be measured in pFF. However, despite the antibodies having been stated as being either proved to work or predicted to work on porcine samples and the concentrated dilutions tested, the antibodies chosen for complement component 4 binding protein α, inter-α-trypsin inhibitor H1, tropomyosin

1 and apolipoprotein E did not result in a visible band on the western blot. This may be due to the dynamic nature of the pFF samples, the antibodies' lack of specificity, the fact that the samples were reduced or that the protein abundance was too low to be detected.

Candidate	**Primary Antibody**			**Secondary Antibody**		
	Details	*Supplier (Product Code)*	*Dilution*	*Details*	*Supplier (Product Code)*	*Dilution*
Complement component 4 binding protein α	Monoclonal Mouse IgG	Abcam (ab167357)	1/500; 1/1000; 1/2000	Donkey AntiMouse IgG H&L (Alexa Fluor® 790)	Abcam (ab175782)	1/100,000
Inter-α trypsin inhibitor H1		Abcam (ab108071)	1/500; 1/1000; 1/2000			
Apolipoprotein A4	Polyclonal Rabbit IgG	LS-Bio (LS-C292890)	1/2000	Goat AntiRabbit IgG H&L (Alexa Fluor® 680)	Abcam (ab175773)	1/100,000
Apolipoprotein M		LS-Bio (LS-C374414)	1/400			
Ceruloplasmin		Abcam (ab110449)	1/2000			
Clusterin		Cloud Corp (PAB180Po01)	1/2000			
Lipopolysaccharide binding protein		LS-Bio (LS-C372726)	1/400			
Plasminogen		Biorbyt (orb13651)	1/100			
Tropomyosin 1		Abcam (ab55915)	1/500; 1/1000; 1/2000			
Apolipoprotein E	Polyclonal Goat IgG	Santa Cruz (sc-31821)	1/200; 1/500; 1/1000	Donkey AntiGoat IgG H&L (Alexa Fluor® 680)	Abcam (ab175776)	1/100,000
Fibrinogen β		LS-Bio (LS-B11267)	1/15000			

Table 3.1. Candidates that were analysed by western blot and the primary and secondary antibodies used.

Apolipoprotein A4, apolipoprotein M, ceruloplasmin, clusterin, lipopolysaccharide binding protein, plasminogen and fibrinogen β had antibodies that worked at concentrations listed on the table. The antibodies for the other candidates did not result in a visible band on the western blot (most likely due to lack of specificity) and the dilutions listed for these candidates were the concentrations attempted but did not result in a visible band.

3.2.6. Measurement of expression

This method is described fully in Eaton *et al.*, (2013). Gels and membranes were scanned with the Licor Odyssey scanner and Image Studio software (Licor Biosciences). Brightness and contrast settings were set to make bands observable. On the western blots, the intensities produced by the protein bands of interest in each sample were measured (**Figure 3.1a**). On the gels, the intensities of whole sample lanes were recorded as well as a "Blank" well which was set as the zero reading to reduce the amount of background interference (**Figure 3.1b**). On the gels, the intensity of each sample was divided by the mean intensity of all the samples on that gel to obtain a ratio for each sample. Each intensity on the western blot was then divided by the ratio from the same coreesponding sample on the gel to give a normalised intensity for each sample. The normalised intensities of the pFF samples were then further normalised relative to the plasma intensity prior to statistical analysis.

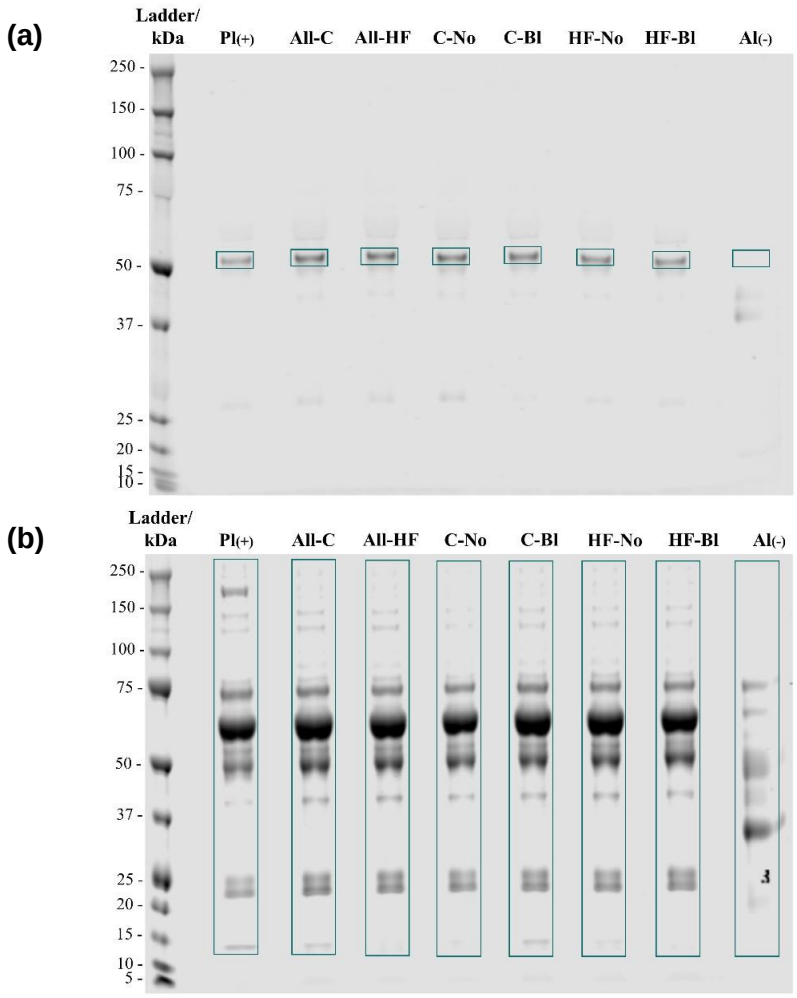


Figure 3.1. Representative images of a western blot incubated in anti-lipopolysaccharide binding protein (1/400) and its corresponding electrophoresis gel.

The images were taken with the Licor Odyssey scanner. The intensity of spots on the western blot (a) at 53 kDa, indicating lipopolysaccharide binding protein, was measured in arbitrary units (AU). The corresponding intensities on the stained gel (b) were used to normalise the intensities on the western blot. Proteins on the gel were stained with Instant Blue Protein Stain. Ladder was Precision Plus Protein Dual Xtra Standards. Abbreviations: Pl(+) = porcine plasma positive control; All-C = all control; All-HF = all high fibre; C-No = control no blastocyst; C-Bl = control blastocyst; HF-No = high fibre no blastocyst; HF-Bl = high fibre blastocyst; Al(-) = porcine allantoic fluid negative control.

3.2.7. Enzyme-linked immunosorbent assay for apolipoprotein A4

An enzyme-linked immunosorbent assays (ELISA) was carried out on individual pFF samples from all 40 experimental pigs. This was carried out using the Pig Apolipoprotein A4 ELISA Kit (Abbexa, Innovation Centre, Cambridge Science Park, Cambridge, CB4 0EY, United Kingdom) and was carried out as per the instructions provided. The plate was read using the Viktor3 Plate Reader (Perkin Elmer, Llantrisant Business Park, Unit A, Llantrisant, CF72 8YW, UK) at 450 nm. The predicted concentration was between 15–40,000 ng/mL, which was adapted from the human plasma concentration of apolipoprotein and was within the working range of the assay (Percy *et al.*, 2014). However, the absorbances for pFF were below the absorbances for 0 ng/mL (negative control). Therefore, no data could be retrieved from this experiment.

3.2.8. Statistical analyses

Three technical repeats of the western blot protocol were carried out for each target protein. Therefore, three normalised expression measurements for each sample pool, relative to the expression in plasma, were obtained for each candidate. All statistical tests were carried out using Minitab 17 Statistical Software. Anderson-Darling normality tests were carried out on the expression measurements. Any that were not normal, were analysed for outliers using the Grubb's test. If the Grubb's test did not identify outliers or if more than five outliers were identified, the normalised expression values were either $\log^{10}$-transformed, squared, square-rooted, cubed or cube-rooted. Anderson-Darling tests and Grubb's tests were repeated, and if none of the transformations yielded normal distributions, the data were regarded as being nonparametric. For each candidate protein, normalised expression values from All-C pFF were compared with the expression values from All-HF pFF, using either twosample t-tests or Mann-Whitney U-tests, depending on whether the measurements were parametric or nonparametric. The normalised expression values of each protein from the C-No, C-Bl, HF-No and HF-Bl pooled pFF samples were analysed with either General Linear Model analysis of variance (ANOVA) or Kruskal-Wallis tests.

Additionally, post-hoc pair-wise comparisons between individual pools were compared using either Student t-tests or Mann-Whitney U-tests.

3.3. Results

3.3.1. Seven candidates were successfully analysed by western Blot

In the studies described in this chapter, quantitative western blots were carried out on 11 candidate proteins. This was to compare the relative expression of the proteins between samples to observe whether they agreed with the relative expressions detected in the proteomic analyses described in chapter 2. The western blots were carried out with the same samples as the proteomic analysis but were not depleted with the Proteominer enrichment kit. Of the 11 candidates, seven had antibodies that produced a visible band on the western blot; the antibodies tested for apolipoprotein E, complement component 4 binding protein α, inter-α-trypsin-inhibitor and tropomyosin 1 did not result in a detectable band on the western blots. The seven candidates with working antibodies were apolipoprotein A4, apolipoprotein M and plasminogen. In addition to these, western blots for plasminogen displayed an extra band (**Figure 3.2**) which meant that the antibody for plasminogen was also able to detect plasmin (appears at approximately 51 kDa), the biologically active form of plasminogen (molecular weight 91 kDa).

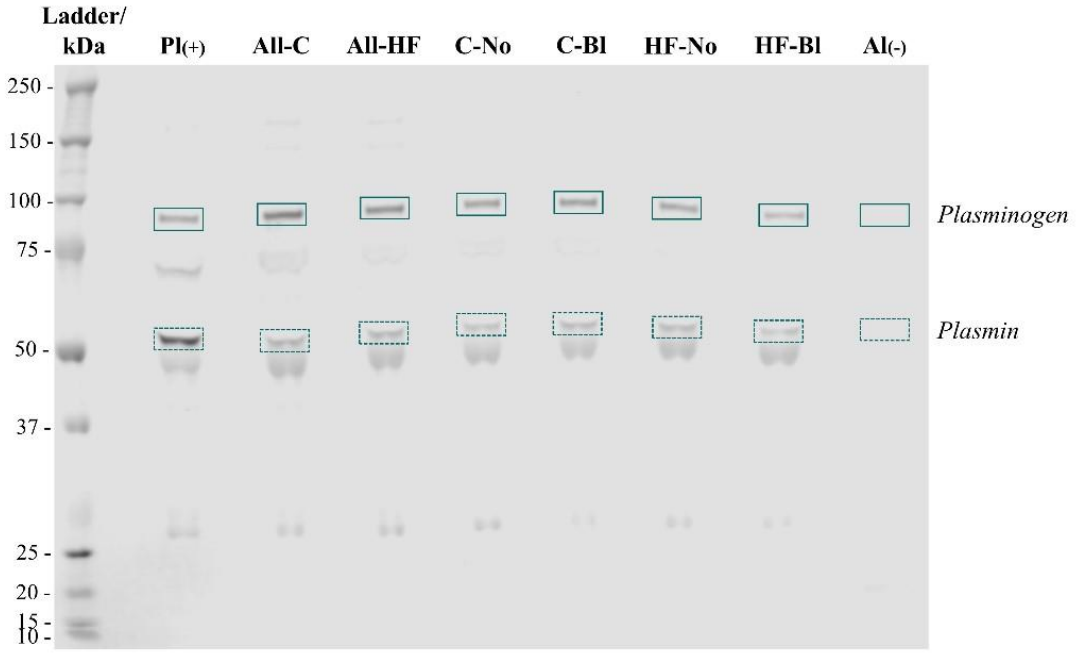


Figure 3.2. Representative image of a western blot incubated with rabbit anti-plasminogen primary antibody (Biorbyt) and Alexa Fluor 680 donkey anti-rabbit IgG H&L secondary antibody (Abcam).

The western blot shows bands for plasminogen highlighted in the square boxes at 91 kDa and plasmin highlighted in the dashed square boxes at 51 kDa in each sample pool. Ladder was Precision Plus Protein Dual Xtra Standards. Abbreviations: Pl(+) = porcine plasma positive control; All-C = all control; All-HF = all high fibre; C-No = control no blastocyst; C-Bl = control blastocyst; HF-No = high fibre no blastocyst; HF-Bl = high fibre blastocyst; Al(-) = porcine allantoic fluid negative control.

3.3.2. Pooled follicular fluid from high fibre fed pigs have lower plasminogen expression compared to follicular fluid from control fed pigs

The antibodies that were able to produce a visible band on the western blot were used in quantitative western blot experiments, which were carried out with triplicate technical repeats for each pFF sample. Therefore, a mean expression was calculated for each protein target in each sample, whereby the n=3 (technical repeats). This method of quantification was an established protocol within the laboratory and is described fully in Eaton *et al.,* (2013) and sections 3.2.6 and 3.2.8. Additionally, the mean expression of each protein in each pFF sample was calculated relative to the expression of that protein in plasma. Two sample t-tests were carried out between the mean expression of each protein in the pFF of control-fed pigs and pFF of high fibre-fed pigs. There were no statistically significant differences between pFF concentrations of apolipoprotein A4, apolipoprotein M, ceruloplasmin, clusterin, fibrinogen β, lipopolysaccharide binding protein, plasmin and combined plasmin and plasminogen (**Table 3.2**). This was in agreement with the Set 2 proteomic analysis. However, the mean expression of plasminogen was lower in All-HF pFF compared to All-C pFF ($p=0.04$), which differed from the proteomic analyses where there were no differences detected between the sample pools.

Candidates	Mean normalised intensity (AU) relative to plasma (±SEM)		P-value
	All-C	All-HF	
Apolipoprotein A4	132.04 (11.91)	129.69 (5.69)	0.88
Apolipoprotein M	102.04 (7.44)	94.72 (9.40)	0.59
Ceruloplasmin	93.91 (6.62)	91.01 (4.78)	0.75
Clusterin	136.62 (6.83)	151.72 (3.10)	*0.19
Fibrinogen β	46.58 (3.49)	40.31 (5.48)	0.41
Lipopolysaccharide binding protein	146.87 (3.84)	136.21 (7.36)	0.29
Plasmin	24.79 (0.36)	23.93 (3.15)	0.81
Plasminogen	131.37 (5.76)	105.90 (4.91)	0.04
Plasmin and Plasminogen	60.16 (2.91)	53.06 (3.73)	0.23

Table 3.2. The mean expression of each candidate in follicular fluid from control and high fibre pigs.

The mean expression of each validation candidate that was analysed, relative to the expression of that candidate in (a reference) porcine plasma sample, along with the standard error of the mean (SEM) in follicular fluid of pigs fed either a control diet (All-C) or a high fibre diet (All-HF), given in arbitrary units (AU). Statistical significance was defined as $p \leq 0.05$ tested as determined by either two-sample t-test or *Mann-Whitney U test.

3.3.3. The increase in expression of apolipoprotein A4, apolipoprotein M and plasmin and the effect of *in vitro* fertilisation outcome on clusterin

General linear model ANOVA (or Kruskal-Wallis) analyses were carried out to compare the relative expression values for each candidate protein in the C-No, C-BL, HF-No and HF-Bl sample pools to identify effects of diet, associations with IVF outcome, or any interactions between diet and IVF outcome. Further pair-wise posthoc comparisons were made between the different sample pools to confirm any relationships between them using either student t-test or Mann-Whitney U tests.

There were no effects of diet or IVF outcome, nor were there any interactions between diet and IVF outcome associated with the expression of apolipoprotein A4, apolipolipoprotein M, ceruloplasmin, fibrinogen β, lipopolysaccharide binding protein, plasminogen and combined plasmin and plasminogen. Pair-wise comparisons identified no statistically significant differences between pFF concentrations of ceruloplasmin (**Figure 3.3a**), fibrinogen β (**Figure 3.3b**), lipopolysaccharide binding protein (**Figure 3.3c**), plasminogen (**Figure 3.3d**) and combined plasmin and plasminogen.

The mean±standard error in arbritary units (AU) of the mean (SEM) expression of apolipoprotein A4 was higher in pFF of control fed pigs associated with blastocyst formation (171.43±12.22 AU) compared to pFF of control-fed pigs without a blastocyst (117.92±10.89 AU, p=0.047, **Figure 3.4a**). The same relationship was observed with apolipoprotein M (105.51±4.70 AU; 79.92±4.49 AU, p=0.029), except that the expression in control-fed pigs associated with blastocyst formation was also higher than that of pFF of high fibre pigs with (86.95±2.61 AU, p=0.041) and without blastocyst formation (79.41±7.04 AU, p=0.054, **Figure 3.4b**). An association between IVF outcome and the protein expression of clusterin was identified (p=0.016, **Figure 3.4c**) by Kruskal-Wallis test. However, the statistical significance was not confirmed by the Mann-Whitney U tests between C-No versus C-Bl expression (p=0.081) or between HF-No versus HF-Bl expression (p=0.191). An interaction between diet and IVF outcome was associated with the protein expression of plasmin

(p=0.008, **Figure 3.4d**). Subsequent pair-wise comparisons confirmed this relationship and identified the lower expression of plasmin in pFF of high fibre-fed pigs whose oocytes formed a blastocyst after IVF (18.26±1.02 AU) compared to pFF of control-fed pigs whose oocytes also formed a blastocyst (22.71±0.86 AU, p=0.045).

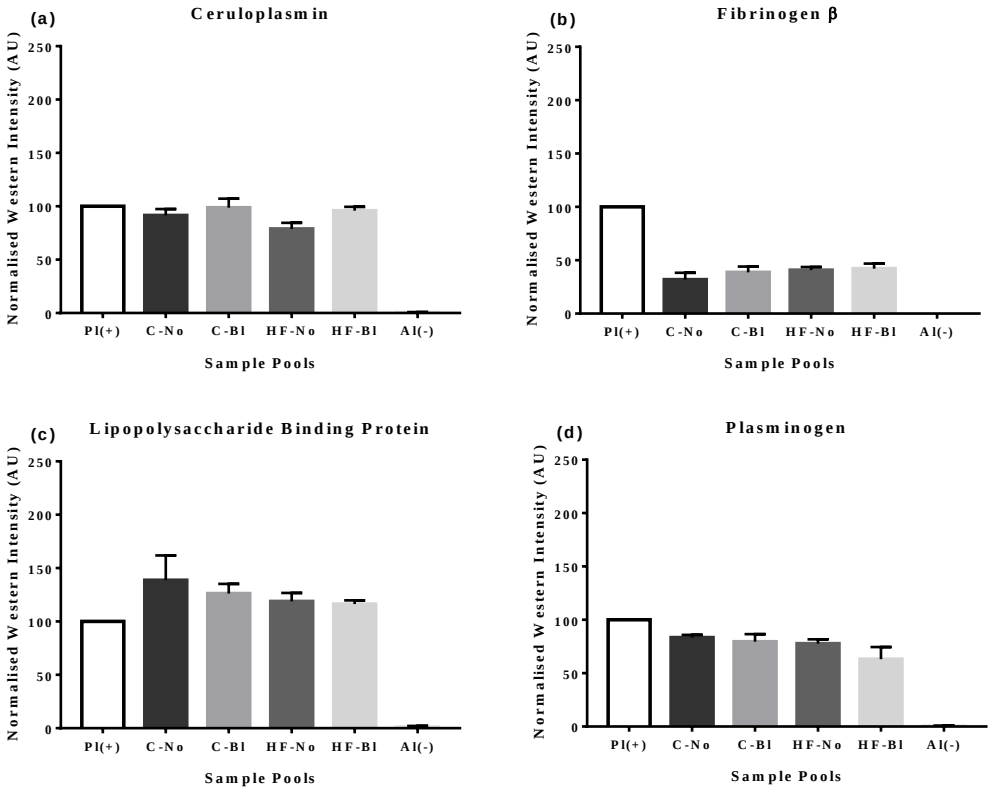


Figure 3.3. The bar charts of protein candidates that were not significantly different between sample pools.

The bar charts illustrate the mean±standard error of the mean (SEM) expression of (a) ceruloplasmin, (b) fibrinogen β, (c) lipopolysaccharide binding protein and (d) plasminogen in pooled follicular fluid of control-fed gilts with oocytes that formed blastocysts (C-Bl) after *in vitro* fertilisation (IVF) and those that did not (C-No), as well as pFF of high fibre-fed gilts with oocytes that formed blastocysts (HF-Bl) after IVF and those that did not (HF-No). The expression of each protein is relative to the expression of that protein in porcine plasma in arbitrary units (AU).

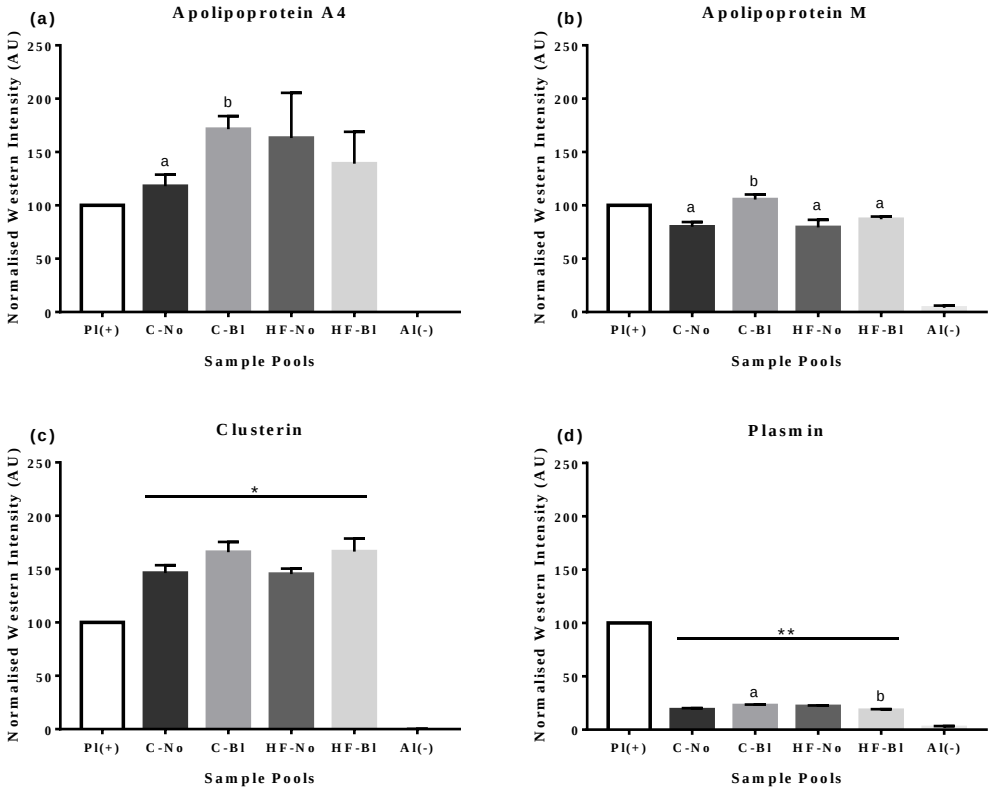


Figure 3.4. The bar charts of protein candidates with significantly different expression between sample pools.

The bar charts illustrate the mean±standard error of the mean (SEM) expression of (a) apolipoprotein A4, (b) apolipoprotein M, (c) clusterin (d) plasmin in pooled follicular fluid of control-fed gilts with oocytes that formed blastocysts (C-Bl) after *in vitro* fertilisation (IVF) and those that did not (C-No) as well as pFF of high fibre-fed gilts with oocytes that formed blastocysts (HF-Bl) after IVF and those that did not (HF-No). The expression of each protein is relative to the expression of that protein in porcine plasma in arbitrary units (AU). General linear model ANOVA or Kruskal-Wallis tests on all the samples. *Kruskal-Wallis test revealed an overall effect of IVF Outcome ($p \leq 0.05$). **ANOVA revealed an interaction between diet and IVF outcome ($p \leq 0.01$). Bars with different subscripts “a” and “b” were significantly different ($p \leq 0.05$) as determined by the Student t-tests.

3.3.4. The expression levels on the western blot were more in agreement with the expression levels detected in the Set 2 proteomic analysis

In chapter 2, section 2.4.2, it was suggested that Set 2 data was more accurate than Set 1 data. The western blots appear to be in agreement with this. In the Set 1 proteomic analysis (**Table 3.3**), the protein expression of three of the candidates were upregulated (apolipoprotein A4, clusterin, fibrinogen β) and two were downregulated (ceruloplasmin, lipopolysaccharide binding protein) in pFF of high fibre-fed pigs compared to pFF of control-fed pigs. However, in Set 2 proteomic analysis, the protein expression of all these candidates, along with apolipoprotein M, were not differentially expressed which was confirmed by the western blots. Plasminogen was not shown to be differentially expressed between pFF of pigs fed the different diets in either Set 1 or Set 2 analysis, but the western blot showed that it was downregulated in pFF of high fibre-fed pigs.

In the pFF of control-fed pigs, most of the candidates were downregulated in samples associated with blastocyst formation compared to no blastocysts in the Set 1 analysis, whilst the opposite was shown in Set 2. The western blots confirmed the up-regulation of three of these (the apolipoproteins) as shown in Set 2 and the rest were not differentially expressed (one in agreement with Set 1 analysis and one in agreement with Set 2 analysis). In the pFF of high fibre-fed pigs, both Set 1 and Set 2 proteomic analyses indicated an up-regulation of the protein candidates in HF-Bl pFF compared to HF-No pFF, but this was only confirmed with clusterin by the western blots.

When only the fold changes were taken into account, again the majority of the ratios in Set 2 datasets were comparable to the western blot ratios or were expressed in the same direction (both lower than 1.00 or both higher than 1.00). The two proteins that were not expressed in the same direction between Set 2 data and the western blots were lipopolysaccharide binding protein and plasminogen.

Candidate	Direction of expression (expression ratio)								
	All-C versus All-HF			C-No versus C-Bl			HF-No versus HF-Bl		
	Set 1	Set 2	Western	Set 1	Set 2	Western	Set 1	Set 2	Western
Apolipoprotein A4	Up (1.77)	NDE (0.91)	NDE (0.98)	Down (0.76)	Up (1.41)	Up (1.43)	Up (1.28)	Up (1.33)	NDE (0.85)
Apolipoprotein M	x	NDE (0.82)	NDE (0.93)	Down (0.65)	Up (1.64)	Up (1.32)	Up (2.63)	Down (0.79)	NDE (1.09)
Ceruloplasmin	Down (0.35)	NDE (0.80)	NDE (0.97)	x	NDE (0.90)	NDE (1.08)	Up (1.33)	Up (1.62)	NDE (1.22)
Clusterin	Up (1.49)	NDE (1.01)	NDE (1.11)	Down (0.79)	Up (1.96)	*Up (1.13)	Down (0.78)	Up (1.49)	*Up (1.15)
Fibrinogen β	Up (1.30)	NDE (0.89)	NDE (0.87)	Down (0.76)	Up (1.43)	NDE (1.22)	x	Up (1.47)	NDE (1.03)
Lipopolysaccharide Binding Protein	Down (0.16)	NDE (1.18)	NDE (0.93)	x	Up (1.45)	NDE (0.91)	x	Up (3.17)	NDE (0.98)
Plasminogen	NDE (1.19)	NDE (0.98)	Down (0.81)	NDE (0.95)	Up (1.62)	NDE (0.95)	Up (1.41)	Up (1.71)	NDE (0.81)

Table 3.3. The list of validation candidates that were analysed by quantitative western blot and the direction of expression compared to the proteomic analyses.

Protein candidates were either upregulated (pink), downregulated (blue), not differentially expressed (NDE, grey) or not detected (x, white), in pooled follicular fluid of all the high fibre-fed pigs (All-HF) or control-fed (C-Bl) and high fibre-fed (HF-Bl) pigs whose oocytes produced a blastocyst following *in vitro* fertilisation, compared to all control-fed pigs (All-C) or control-fed (C-No) and high fibre-fed (HF-Bl) pigs without a blastocyst respectively. Brackets indicate the expression ratios derived from All-HF/All-C protein levels, C-Bl/C-No protein levels, HF-Bl/HF-No protein levels. Differentially expressed proteins in the proteomic analyses had ratios greater than 1.25 or lower than 0.8, but differences were statistically determined from the western blots.

3.4. Discussion

In this chapter, quantitative western blots successfully validated the differential expression of four candidate proteins with likely or known reproductive functions in pFF. These proteins were involved with inflammatory processes relating to ovulation and their differential expression indicates a lengthening of the maturation process as well as a delay in ovulation.

3.4.1. The differences between detection by western blotting and mass spectrometry

The protein expression for most of the candidate proteins were confirmed for the Set 2 analysis, making the results of the Set 2 proteomic analysis more reliable. Any differences between the proteomic analyses and the western blots could be attributed to the differences between the sample types. The proteomic analyses were carried out on Proteominer depleted samples whilst the western blots were carried out on unaltered pFF pools. This was because there was no need to remove high abundance proteins for western blot detection and the aim of the western blots was to assess differences in protein expression in un-manipulated pFF. Despite being a powerful depletion kit, it was difficult to know exactly which proteins the Proteominer targets. Whilst the proteomic analyses aimed to identify potential biomarkers present at low levels, for the purposes of validating the proteomic analyses, the protein candidates needed to be present at high enough levels in non-depleted samples to be detected by western blotting. Therefore, it is possible that the Proteominer had targeted some of the candidate proteins that were more abundant such as lipopolysaccharide binding protein.

Curiously, many of the most recent published proteomic studies that use the Proteominer kit did not include any validation by either western blots or ELISAs, although most of these studies came from the same research groups (Altomare *et al.*, 2016; Geary *et al.*, 2016; Longo *et al.*, 2016; Tacoma *et al.*, 2016, 2017a, b, De Jesus *et al.*, 2017a, b; Manfredi *et al.*, 2017). However, there have been studies that have confirmed the expression of two and four candidate proteins following Proteominer

depletion and protein detection by liquid chromatography tandem mass spectrometry of human serum samples and rat testicular interstitial fluid respectively (de Seny *et al.*, 2016; Stanton *et al.*, 2016). These were comparable numbers of validated proteins with differential expression as the number of validated proteins in this study, and there were no details of the candidates that did not validate in those papers.

3.4.2. The high fibre diet was associated with lower concentration of plasmin and plasminogen in porcine follicular fluid which may be indicative of a delay in ovulation

In preovulatory follicles, luteinising hormone (LH) stimulates a cascade of proteolytic enzymes including plasminogen/plasmin that degrade the perifollicular matrix, leading to the decomposition of the meshwork of collagen fibres that provide strength to follicular wall (Tsafriri, 1995). Quantitative western blotting on non-depleted pFF samples identified that pFF of high fibre pigs had lower plasminogen expression compared to pFF of control-fed pigs. This suggests that fewer of the follicles from the high fibre-fed pigs were ready to ovulate at the time of slaughter compared to their control counterparts since they did not contain as much of the pre-cursor protein that is involved in weakening the follicular wall. Additionally, prior to ovulation (day 0–2 of oestrous cycle), circulating concentrations of oestradiol peak (day 19), leading to a surge in LH (day 21). In high fibre pigs, circulating concentrations of oestradiol were lower on days 17 and 18 of the oestrous cycle, whilst an increase in LH pulses were observed at day 18 compared to control-fed pigs (Ferguson *et al.*, 2007). This indicates a lengthening of the maturation process due to the suppression of the negative feedback on the hypothalamic pituitary gonadal axis. This lengthening means that at the time of slaughter (day 19 of oestrous cycle) the high fibre pigs were less likely to be at the precipice of ovulation. Measuring circulating levels of LH to detect the LH surge prior to sample collection may increase the likelihood of the interval between pFF collection and ovulation were more uniform.

The expression level of the active plasmin was not different between high fibre and control samples overall, but the samples from high fibre-fed pigs whose oocytes formed blastocysts was lower than in samples from control pigs whose oocytes formed blastocysts. This indicates that despite the lack of the proteolytic protein, blastocysts

were still able to be formed. However, despite the increased number of cells in blastocysts from high fibre-fed pigs, the diet was also associated with fewer blastocysts formed overall (Ashworth *et al.*, 2008). Therefore, this increase in maturation could also be attributed to the lower concentration of proteins required for the initiation of blastocyst formation. Additionally, there were no differences confirmed in fibrinogen β protein expression between the pFF samples, although this does not mean that plasminogen and fibrinogen do not directly act in synergy as the differences in fibrinogen may only occur after ovulation.

Curiously, feeding a high fibre diet from various sources to rabbits, baboons and adults of various ages has also been associated with a reductions in circulating plasminogen activator inhibitor-1, the inhibitor of the enzyme that catalyses the activation of plasminogen into plasmin (Venter *et al.*, 1997; Kristensen and Bügel, 2011; Rizkalla *et al.*, 2012; Roberts *et al.*, 2013; Valero-Muñoz *et al.*, 2014; Miller *et al.*, 2015; Ampatzoglou *et al.*, 2016). The differences in plasminogen activator inhibitor-1 could imply that the alteration in plasminogen and plasmin expression were dependent on the LH surge as opposed to dis-regulation of the plasminogen activator protein, which accounts for most of the plasmin activity in the ovary (Ny *et al.*, 1997).

3.4.3. An increase was observed in apolipoproteins associated with blastocyst formation between follicular fluid of pigs whose oocytes produced a blastocyst with follicular fluid of pigs without a blastocyst

Clusterin

This study identified a significant association of IVF outcome with clusterin (apolipoprotein J) expression, with pFF pools associated with blastocyst formation having higher expression levels. Increased clusterin expression has not been shown to be beneficial towards ovarian cells as blastocysts from diabetic rats had more clusterin transcripts than blastocysts from normal rats, and half the cells that expressed clusterin mRNA exhibited signs of nuclear fragmentation (Pampfer *et al.*, 1997). Additionally, clusterin protein and mRNA expression was observed in granulosa cells of atretic rat follicles, which increased with the level of apoptosis, whereas granulosa cells of

healthy rat follicles did not express clusterin (Zwain and Amato, 2000). Increased levels of clusterin in the microenvironment also appears to be detrimental with cytoplasmic over-expression of clusterin detected in human ovarian carcinomas which also inversely correlated with tumour apoptosis (Xie *et al.,* 2005). Additionally, levels of clusterin transcripts were higher in bovine cumulus cells following *in vitro* maturation (IVM) in hormone-free Tissue Culture Medium 199 compared to cumulus cells matured in medium enriched in gonadotrophins and growth factors (Salhab *et al.*, 2011). However, clusterin mRNA expression was shown to increase in bovine cumulus cells at 10 hours of IVM, indicative of its role during metaphase-I of meiosis (Salhab *et al.,* 2011). Additionally, the level of transcripts does not represent total clusterin levels and it has recently been discovered that the different chains of clusterin act differently; treatment of mouse myoblast cells with full length clusterin lowered lipid levels in those cells whilst the β-chain alone doubled lipid levels (Matukumalli *et al.*, 2017).

Whether this association has biological significance with regards to oocyte maturation remains uncertain as gilt oocytes themselves do not contain clusterin transcripts, whereas boar spermatozoa do (Kempisty *et al.,* 2008). Zygotes and two-cell stage embryos produced from the fertilisation of oocytes with spermatozoa also contain clusterin transcripts, implying that the spermatozoa delivered the clusterin mRNAs to the oocyte (Kempisty *et al.*, 2008). Similar observations of clusterin transcripts were observed with human spermatozoa, hamster oocytes and human-hamster zygotes (Ostermeier *et al.*, 2004). However, clusterin levels in bovine oocytes were measured and were found to have decreased by half after IVM and the authors suggested that clusterin could have been synthesized in the cumulus cells and transferred to the oocyte via gap junctions during oocyte development (Berendt *et al.*, 2009). Therefore, it is safe to infer that the changes in clusterin levels observed in this study were not due to changes in transcription of the protein by the oocyte, but that the levels were dependent on the transfer of circulating clusterin from plasma or secretions from granulosa cells. Additionally, the increase of clusterin in the microenvironment is in this case beneficial and perhaps required for blastocyst development.

Apolipoprotein A4 and apolipoprotein M

The western blots have also shown that the protein expressions of apolipoprotein A4 and apolipoprotein M were higher in pFF of control-fed pigs with blastocysts compared to control-fed pigs with no blastocysts but was not different between any high fibre samples. This is partly due to the large amount of variation in apolipoprotein A4 expression within the HF-No and HF-Bl pFF pools. However, the expression of apolipoprotein M was also higher in control-fed pigs with blastocysts compared to both high fibre sample associated with and without blastocysts. This may indicate a deregulatory process of the diet on circulating lipoprotein levels.

Differences in apolipoprotein A4 protein expression appear to have negative consequences as it is a serum biomarker for ovarian cancer and for malignant tumour differentiation in women with elevated serum cancer antigen 125 (Li *et al.*, 2012a; Lorkova *et al.*, 2012; Rauniyar *et al.*, 2017). Additionally, apolipoprotein A4 protein expression has been shown to have increased in serum and plasma of patients with malignant ovarian cancer patients compared to benign ovarian cancer patients (Dieplinger *et al.*, 2009; Timms *et al.*, 2014).

The role of apolipoprotein A4 in lipid and cholesterol efflux also influences the progression of diseases associated with lipids as protein expression increased in follicular fluid from patients with polycystic ovaries compared with fluid from normal controls whilst ovarian apolipoprotein A4 gene expression was downregulated in obese mice compared to lean mice (Kim *et al.*, 2013; Vidotti *et al.*, 2015). The role of apolipoprotein M in ovaries is not well-established but its transcripts have been detected in mouse embryos from day 7.5 to day 18.5 and plasma concentrations of apolipoprotein M in pregnant women increased during pregnancy (Zhang *et al.*, 2004; Ahnström *et al.*, 2010).

As with plasmin and plasminogen, the differences in levels of apolipoprotein A4 and apolipoprotein M between samples may be associated with the lower oestradiol concentration and the increase in LH pulses associated with the high fibre samples. Evidence suggests that the expression of apolipoprotein A4 and apolipoprotein M are

likely directly dependent on oestradiol level and for apolipoprotein M, this is through the oestrogen receptor (Wei *et al.*, 2011; Shen *et al.*, 2014). Due to the delay in ovulation, the benefit may be the increased time for oocytes to mature, but there may be detrimental effects where the high fibre diet disrupts or delays important inflammatory or metabolic mechanisms involving apolipoproteins.

There are other potential links between the plasminogen cascade and apolipoprotein function, as feeding baboons soluble fibre for eight weeks lowered plasminogen activator inhibitor-1 along with total cholesterol and circulating free fatty acid levels (Venter *et al.*, 1997). LH and human chorionic gonadotropin (hCG) also stimulates eicosanoids including prostaglandins which regulate the proteolytic cascade involving plasminogen and plasmin (Tsafriri, 1995). This consolidates the potential relationship between the high fibre diet, plasminogen/plasmin cascade and circulating lipid levels.

3.4.4. Limitations

One of the negative aspects of working with pig samples compared to mouse or human samples is the difficulty in obtaining functional antibodies. In this study, out of the 11 protein candidates selected, seven had antibodies that worked, thereby eliminating the possibility of confirming the other candidates and for potential further insight this would have provided. There are also limitations of comparing pooled pFF instead of measuring concentrations of individual animals. An ELISA for apolipoprotein A4 was carried out on individual pFF samples but the assay failed to detect any apolipoprotein A4. Without limitations on samples and time, further western blots could have been carried out to measure the expression levels of each candidate in pFF samples from individual animals in three samples from each feeding group and IVF group.

3.4.5. Conclusions and next steps

The lower plasminogen expression in pFF samples from high fibre-fed pigs compared to pFF from control-fed pigs which was further represented with the decreased expression levels of plasmin between the pigs fed the different diets but whose oocytes produced a blastocyst. This suggests that plasmin and its plasminogen precursor may

be biomarkers for oocyte maturation stage with the lower levels being indicative of a delay in ovulation. Lower plasmin/plasminogen expression and delayed ovulation in high fibre-fed gilts could be implicated in the decrease in blastocyst rate. Along with plasmin and plasminogen, the differential expression of apolipoprotein A4, apolipoprotein M and clusterin was detected. The differential expression of clusterin between samples associated with blastocyst development and samples without a blastocyst was indicative of the requirement of this protein. With the exception of clusterin, the differential expression of the apolipoproteins between pFF associated with blastocyst formation and pFF without blastocysts was not observed in high fibre samples, leading one to speculate that this process was either disrupted or delayed.

The prospect of a disruption in lipoprotein levels due to a high fibre diet is not unique as it is well known that dietary fibre reduces low-density lipoprotein cholesterol and lipid levels (Surampudi *et al.*, 2016). Therefore, due to the interest in the potential effects of the high fibre diet on pFF levels of lipids, a targeted metabolomic analysis was carried out for fatty acids, which is described in chapter 4.

4. Targeted Metabolomic Analysis of Porcine Follicular Fluid

4.1. Introduction

The proteomic analyses described in chapters 2 and 3 revealed the differential expression of apolipoprotein A4 and apolipoprotein M between porcine follicular fluid (pFF) of control-fed pigs whose oocytes produced a blastocyst following *in vitro* fertilisation (IVF) and pFF of control-fed pigs whose oocytes did not produce a blastocyst. Conversely, there was no significant difference between high fibre-fed pigs whose oocytes produced a blastocyst and high fibre-fed pigs whose oocytes did not. Additionally, the levels of apolipoprotein M were significantly higher in control-fed pigs with a blastocyst compared to high fibre-fed pigs of both IVF outcomes, which may indicate a disruptive effect of the diet on apolipoprotein-mediated effects during blastocyst formation. The apolipoproteins that were differentially expressed are involved in cholesterol and lipid efflux and metabolism. Therefore, following the proteomic results, one of the potential next steps was to examine the possible association between the high fibre diet with pFF lipids, in particular fatty acids as they are the major components of lipids.

4.1.1. Alteration in fatty acids in follicular fluid due to diet

The diet consumed by an animal is an important factor in the metabolism of fatty acids and the changes in fatty acid metabolism affects the molecular composition of ovarian follicular fluid (FF). It has been previously shown that gilts fed a diet abundant in ω3fatty acids had pFF enriched with saturated fatty acids (SFAs) such as palmitic acid, as well as reduced polyunsaturated fatty acids (PUFAs) such as linoleic acid (Warzych *et al.*, 2011). Additionally, the supplementation of bovine diets with flaxseed, soybean and fish oils altered the relative amounts of FF fatty acids such as oleic acid, *cis*-9, *trans*-11 conjugated linoleic acid (CLA), palmitoleic acid, and linolenic acid (Childs *et al.*, 2008; Zachut *et al.*, 2010, 2011; González-Serrano *et al.*, 2015). This is

especially interestingly as ruminants have digestive microorganisms that hydrogenate PUFAs into SFAs and monounsaturated fatty acids (MUFAs, Vernon, 1980).

4.1.2. Research aims

The aim of this study was to ascertain whether the high fibre diet affected the concentration of fatty acids in pFF and whether these alterations were further associated with IVF outcome/fertility. A targeted metabolomic analysis was carried out to measure the concentration of the most abundant fatty acids in pFF, as determined by Yao *et al.*, (1980). These fatty acids were myristic acid, palmitic acid, stearic acid, arachidic acid, palmitoleic acid, oleic acid, linoleic acid, α-linolenic acid, dihomo-γ-linolenic acid (DGLA), arachidonic acid, adrenic acid and docosapentaenoic acid (DPA). In addition to these fatty acids, the concentrations of erucic acid and behenic acid were also measured as these had previously been shown to be significantly different in a previous study (Bender *et al.*, 2010).

4.2. Materials and Methods

4.2.1. Preparation of fatty acid standards

All chemicals were provided by Cambridge Bioscience (Munro House, Trafalgar Way, Bar Hill, Cambridge, CB23 8SQ) unless specified. Solid palmitic, stearic, myristic, behenic and arachidic acids were weighed and dissolved in either MS-grade methanol (Honeywell, VWR) or MS-grade ethanol (Fluka, Thermo Fisher Scientific) at a suitable solubility (as recommended by the supplier). Oleic, linoleic, erucic, αlinolenic, arachidonic, adrenic acids, DGLA and DPA were provided in aqueous suspension, which were diluted to the appropriate concentrations.

Fatty acid	Lipid numbers	State; mass (mg) or concentration (mg/mL)	Resuspension solvent	Further dilution	Final concentration (mg/mL)
Myristic	C14:0	Solid; 1000	10 mg in 1 mL MeOH	1/10	1
Palmitic	C16:0	Solid; 10000	20 mg in 1 mL MeOH	n/a	20
Stearic	C18:0	Solid; 500	20 mg in 1 mL EtOH	n/a	20
Arachidic	C20:0	Solid; 50	2 mg in 1 mL MeOH	N/A	2
Behenic	C22:0	Solid; 250	2 mg in 1.5 mL EtOH	1/10	0.133
Palmitoleic	C16:1 ω6	Neat oil; 100	894 mg/mL	27.96 µl in 472 µl EtOH	50
Oleic	C18:1 ω9	Aqueous; 500	N/A	1/10	50
Erucic	C22:1 ω9	Aqueous; 250	N/A	1/100	0.5
Linoleic	C18:2 ω6	Aqueous; 500	N/A	1/10	50
α-Linolenic	C18:3 ω3	Aqueous; 250	N/A	1/5	5
DGLA	C20:3 ω6	Aqueous; 100	N/A	1/10	10
Arachidonic	C20:4 ω6	Aqueous; 250	N/A	1/5	50
Adrenic	C22:4 ω6	Aqueous; 100	N/A	1/10	10
DPA	C22:5 ω6	Aqueous; 10	N/A	N/A	10

Table 4.1. List of fatty acids that were analysed and the preparation of their diluted stock solutions.

The fatty acids listed as an aqueous solution were provided in an ethanol resuspension. Further dilutions were carried out using the same diluent that the fatty acid was originally dissolved in or provided in. Abbreviations: N/A = not applicable; MeOH = methanol; EtOH = ethanol; DGLA = dihomo-γ-linolenic acid; DPA = docosapentaenoic acid.

4.2.2. Preparation of margaric acid internal standard

Margaric acid (heptadecanoic, C17:0, Sigma Aldrich) was resuspended in MS-grade methanol, making a 20 mg/mL solution. For each experiment, a diluted solution of 0.26 mg/mL was made (diluted with MS-grade methanol); enough for each diluent and sample so that the final concentration was 0.01 mg/mL. The margaric acid was prepared in the Chromacol glass tubes.

4.2.3. Preparation of dilution series

The neat solution of the dilution series contained each fatty acid to be analysed but at different starting concentrations (**Table 4.2**). Each fatty acid had a different concentration as they are present in pFF at different amounts. The starting concentrations were determined through a mixture of both previously published concentrations from Yao *et al.*, (1980) and qualitative analyses of spare pFF. The most concentrated sample (top diluent) was serially diluted by two-fold dilutions in MSgrade H_2O (Thermo Fisher Scientific) to produce eight calibrant solutions of different concentrations.

Fatty acid	Lipid numbers	Concentration of stock solution (mg/mL)	Volume added to make 1 mL top diluent (µl)	Final concentration of neat solution (mg/mL)
Myristic	C14:0	1	10	0.01
Palmitic	C16:0	20	10	0.2
Stearic	C18:0	20	5	0.1
Arachidic	C20:0	2	20	0.04
Behenic	C22:0	0.133	7.5	0.001
Palmitoleic	C16:1 ω6	50	8	0.4
Oleic	C18:1 ω9	50	4	0.2
Erucic	C22:1 ω9	0.5	2	0.001
Linoleic	C18:2 ω6	50	2	0.1
α-Linolenic	C18:3 ω3	5	8	0.04
DGLA	C20:3 ω6	10	4	0.04
Arachidonic	C20:4 ω6	50	2	0.1
Adrenic	C22:4 ω6	10	4	0.04
DPA	C22:5 ω6	10	4	0.04
MS-grade H_2O	N/A	N/A	909.5	N/A

Table 4.2. List of fatty acids that were analysed and their contribution to the neat solution in the dilution series.

The table lists the fatty acids that were analysed, the concentration of the stock solution (see **Table 4.1**) and the volume of that stock solution required to make the final concentration in the neat (top) solution of the dilution series. The final concentration of each fatty acid in the final column was higher than the predicted concentration range so that the measured concentrations of each sample fitted onto the standard curve. Abbreviations: DGLA = dihomoγ-linolenic acid; DPA = docosapentaenoic acid.

4.2.4. Preparation of samples

The pFF samples used in the metabolomic analysis were the fluids from Ashworth *et al.*, (2008) described in Section 1.2.1. Specifically, the "Pool 1" pFF from 12 animals were used for the analysis; six of the animals were fed the control diet whilst the other six were fed the high fibre diet. Within each of the six, half had oocytes that produced blastocysts following IVF whilst the other half had oocytes that did not produce a blastocyst. Fluid samples were removed from -80 °C, thawed on ice, centrifuged at 5000 xg for 5 minutes and vortexed for ten seconds to mix thoroughly. This was to ensure that there was not any sample left on the lid before vortexing. The samples were separated into two batches which were prepared and analysed on separate occasions. Each sample was analysed in duplicate.

4.2.5. Bligh and Dyer extraction

All reagents were supplied by Thermo Fisher Scientific unless specified. From each sample and standard in the dilution series, 50 µL was dispensed into Clear-view snapcap microtubes (Sigma Aldrich) and 2 µL margaric acid (0.26 mg/mL stock solution) was added to each sample and standard to make a final concentration of 0.01 mg/mL. Metabolites were extracted by the addition of 187.5 µL of 1:2 (v:v) mixture of chloroform:methanol (both MS-grade), 67.5 µL of MS-grade chloroform and 67.5 µL of MS-grade water. After the addition of each solvent, the mixtures were vortexed for 1 minute. The sample and standard mixtures were then centrifuged at 5000 g in an IEC table-top centrifuge for 15 min at room temperature to give a two-phase system (aqueous top, organic bottom). Most of the aqueous top layer was gently removed with a pipette and discarded. The bottom phase was carefully recovered and placed into new Chromacol tubes. The solvent was evaporated on a Dri-Blocks heat block (Techne, VWR) set to 50 °C for 2 hours and each sample was re-dissolved in 50 µL of 80 % (v/v) methanol in water.

4.2.6. Detection by liquid chromatography tandem mass spectrometry

Liquid chromatography (LC) tandem mass spectrometry (MS/MS) was carried out in collaboration with Dr Andrew C. Gill (Roslin Proteomics and Metabolomics Facility). All reagents, equipment and materials were supplied by Thermo Fisher Scientific unless specified. The metabolite extracts analysed on the UltiMate 3000 HPLC systems and the amazon ETD (Bruker, Banner Lane, Coventry, CV4 9GH UK) tandem mass spectrometer. The HPLC column used was ACE UltraCore 2.5 SuperC18 column (75 x 2.1 id, 2.5 µm; Advanced Chromatography Technologies Ltd, 1 Berry Street, Aberdeen, AB25 1HF) and was maintained at a temperature of 40 °C during analysis. Mobile phase "A" was a solution of 50 mM ammonium formate at pH 3.0 whilst mobile phase "B" was a solution of methanol containing 0.1% formic acid. The elution gradient was as follows: 0 min, 10 % B; 1 min, 87 % B; 8 min, 90% B; 10 min 100% B, resulting in a total run time of 12 min. The system was re-established and equilibrated with 10% B for two minutes. The injection volume was 5 µl and the flow rate was 200 µl/min which remained constant. Nitrogen was used as desolvation and nebulising gas and argon as the collision gas. The dry gas flow rate was 8.0 L/min with temperature of 150 °C and the nebulizer was 16.0 psi. The elution was detected using MS/MS in negative electrospray multiple reaction monitoring mode. **Table 4.3** lists the LC-MS/MS conditions required for the detection of each fatty acid. Note that due to the stability of the fatty acids, fragmentation proved to be difficult. Therefore, the parent ions detected with the negative ions produced.

4.2.7. Identification and quantitative analyses

Raw mass spectral data were processed with Hystar PP Version 3.2.44.0 data analysis software (Bruker) to generate peaks lists and quantitation was carried out using Bruker Compass QuantAnalysis 2.0 SP 2 (Bruker Daltonik GmbH, Bremen, Germany).

Fatty acid	Molecular weight (g/mol)	Parent ion (*m/z*)	Retention time (Minutes)	Fragmentation cut-off (*m/z*) and amplitude (*v*)
Margaric	270.45	269.1	7.50	73 and 0.7
Myristic	228.37	227.1	4.50	61 and 0.7
Palmitic	256.42	255.1	6.30	69 and 0.7
Stearic	284.48	283.1	9.00	76 and 0.7
Arachidic	312.53	311.1	10.80	84 and 0.7
Behenic	340.58	339.1	11.80	92 and 0.7
Palmitoleic	254.41	253.1	4.90	68 and 0.7
Oleic	282.46	281.1	6.80	76 and 0.7
Erucic	338.57	337.1	11.00	91 and 0.7
Linoleic	280.45	279.1	5.50	75 and 0.7
α-Linolenic	278.43	277.1	4.60	75 and 0.7
DGLA	306.49	305.1	6.30	82 and 0.8
Arachidonic	304.47	303.1	5.40	82 and 0.7
Adrenic	332.50	331.1	7.15	89 and 0.7
DPA	330.50	329.1	5.90	89 and 0.7

Table 4.3. The optimised tandem mass spectrometry conditions used to detect each fatty acid.

For each fatty acid, a parent ion is produced and detected. The retention time is the time in which the parent ion runs through the column is detected during the 12-minute run time. The collision-induced dissociation (CID) parameters also include the fragmentation cut-off (*m/z*) and amplitude (*v*). Abbreviations: DGLA = dihomo-γ-linolenic acid; DPA = docosapentaenoic acid.

4.2.8. Method optimisation

The methodology for the targeted metabolomic analysis was not set up at the institute. Therefore, a series of optimisations were carried out to develop the protocol described in sections 4.2.4, 4.2.5 and 4.2.6 of this chapter. The first set of optimisations was to ensure that the fatty acids could be accurately detected. This was carried out by analysing each fatty acid standard individually, to determine the optimum retention times and fragmentation conditions for each fatty acid. The accuracy of the measurements was then confirmed through measuring each fatty acid in a dilution series, to confirm the linear result in the measurements. Additionally, other conditions were tested, including the concentrations of methanol and acetonitrile in the mobile phase elution buffers and subsequently the most efficient elution gradient.

The next method to optimise was the extraction of the fatty acids by the Bligh and Dyer method. The method was carried out initially with sterilised plastic pipette tips but the recovery following this method was inaccurate as the standard curves became disrupted. Alternatives were sought after and Nichipet ECO glass pipette tips (Nichiryo, 2760-1 Nishikata, Koshigaya-shi, Saitama 343-0822, Japan) were initially tested. However, these proved difficult to use with the small volumes that were extracted in this study. Therefore, 10 µl LongReach Barrier low binding sterile pipette tips (Sorenson Biosciences Inc., 6507 South 400 West Salt Lake City, Utah 84107), were eventually selected, as these enable the accurate recovery of the small volume of sample and prevented the sample from sticking to it. Different samples and volumes were also tested. Initially, the Bligh and Dyer method was tested on porcine plasma (same as the one used in chapter 3, section 3.2.1), starting at 500 µl, and then 100 µl, 75 µl and 50 µl. Once it was confirmed that extraction and detection worked for the lowest volume (50 µl), the method was tested on spare "Pool 2" pFF (see chapter 2, section 2.2.1). Once the method worked with this, the analysis was ready.

4.2.9. Recording of cleavage rate, blastocyst rate, blastocyst cell number, oestradiol concentration

This was carried out by Dr Elizabeth M. Ferguson at the time of ovary collection, as described in Ashworth *et al.*, (2008). Briefly, the 16 largest follicles were aspirated from the ovaries of pigs fed either the control or high fibre diet on day 19 of their third oestrous cycle. *In vitro* maturation (IVM) of the oocytes was carried out in Tissue Culture Medium 199 supplemented with luteinising hormone and follicle stimulating hormone and the animals' own pFF for 44 hours. Following IVF, embryos were cultured in NCSU-23 medium for 6 or 7 days, and the cleavage rates were recorded during this time. Blastocysts were fixed in Hoescht 33258 and blastocyst rates and cell numbers were recorded. PFF was pooled within animal and oestradiol concentrations were determined by radioimmunoassay. The results of this study are summarised in **Appendix 2**.

4.2.10. Statistical analysis

The pFF samples (n=24) were divided into two sets and each sample set was analysed in duplicate, giving four batches in total (A, B, C and D) where batches A and B assayed the same 12 samples and batches C and D assayed the other 12 samples. Within each batch, each sample (and standard) was analysed twice. Therefore, the accuracy of each individual assay was assessed by calculating the intra-assay coefficient variation (CV) for each fatty acid in each sample. Additionally, for each fatty acid in each sample, the mean concentration and the standard deviation was calculated as well as the inter-assay CV.

All statistical tests were carried out using Minitab 17 Statistical Software. For each fatty acid, concentration measurements were subjected to Anderson-Darling Normality tests. Data that were not normally distributed underwent an outlier tests. If there were no outliers or if removing outliers (maximum of three) did not result in a normal distribution, data were transformed by a series of functions; $\log^{10}$, ln, square root, squared, cubed, to the power of 4–10. General Linear Model analysis of variance (ANOVA) was then carried out on normally distributed data where diet and IVF

outcome were included as factors and batch as a covariate. For data that were not normalised, Kruscal-Wallis tests were carried out between measurements of samples from pigs fed the different diets and associated with different IVF outcomes. An additional post-hoc analysis was carried out with Tukey Pairwise Comparisons. Regression and correlation analyses were carried out between the fatty acid concentrations versus oestradiol concentration, cleavage rate, blastocyst rate, blastocyst cell number, follicle size and FF volume. The regression analyses were carried out twice, first with the fatty acid concentrations as the dependent and second as the independent variable. Note that palmitoleic acid was not included in the regression analyses as the measurements for its concentration could not be normalised.

4.3. Results

4.3.1. Detection of fatty acids and the standard curves produced from the dilution series

In this study, a targeted metabolic approach was used to detect 14 fatty acids in pFF. Optimisation conditions for the metabolic protocol ensured that the detection of these fatty acids was accurate (**Figure 4.1**). However, out of the 14 fatty acids studied, myristic acid, palmitic acid, stearic acid, α-linoleic acid and behenic acid were not able to be accurately measured. This was because the levels of behenic acid were too low for detection, whilst the standard curve for myristic acid, palmitic acid, stearic acid and α-linoleic acid had intercepts that were not y=0 (**Figure 4.2**), indicating the presence of those fatty acids at very low levels in the MS-grade dH_2O, possibly due to contamination from plastics or the glassware. This contamination is not a rare occurrence as others have reported similar problems, with stearic and palmitic acids being the most common contaminants, often attributed to the use of plastic pipette tips and tubes (Lee *et al.*, 2015; Tumanov *et al.*, 2015). Aside from using glassware, it was also suggested that any vials or plastic tips could be rinsed with methanol or the same mobile phase elution solvent to prevent palmitate contamination and for samples to be stored in vials with caps lined with polytetrafluoroethylene (Song *et al.*, 2016; Zhang *et al.*, 2016). However, despite the contamination observed in this study, the concentration of nine fatty acids present in pFF was able to be measured with good standard curves (**Figure 4.3**).

The inter- and intra-assay CVs measured also varied between fatty acids, samples and batches. The fatty acids with the best mean inter-assay CV values included palmitoleic acid, linoleic acid and adrenic acid, all of which were less than 15 %, whilst the fatty acids with the worst included arachidic acid and erucic acid (**Table 4.4**). The mean intra-assay CVs for most of the fatty acids was adequate (less than 10 %), apart from the mean values for oleic acid, erucic acid and arachidonic acid, with intra-assay CVs higher than 20 (**Table 4.4**). The inter- and intra-assay values for individual samples (**Appendices 4–6**) agreed with this observation and revealed that linoleic acid had the most number of samples with an inter-assay CV of less than 15 %.

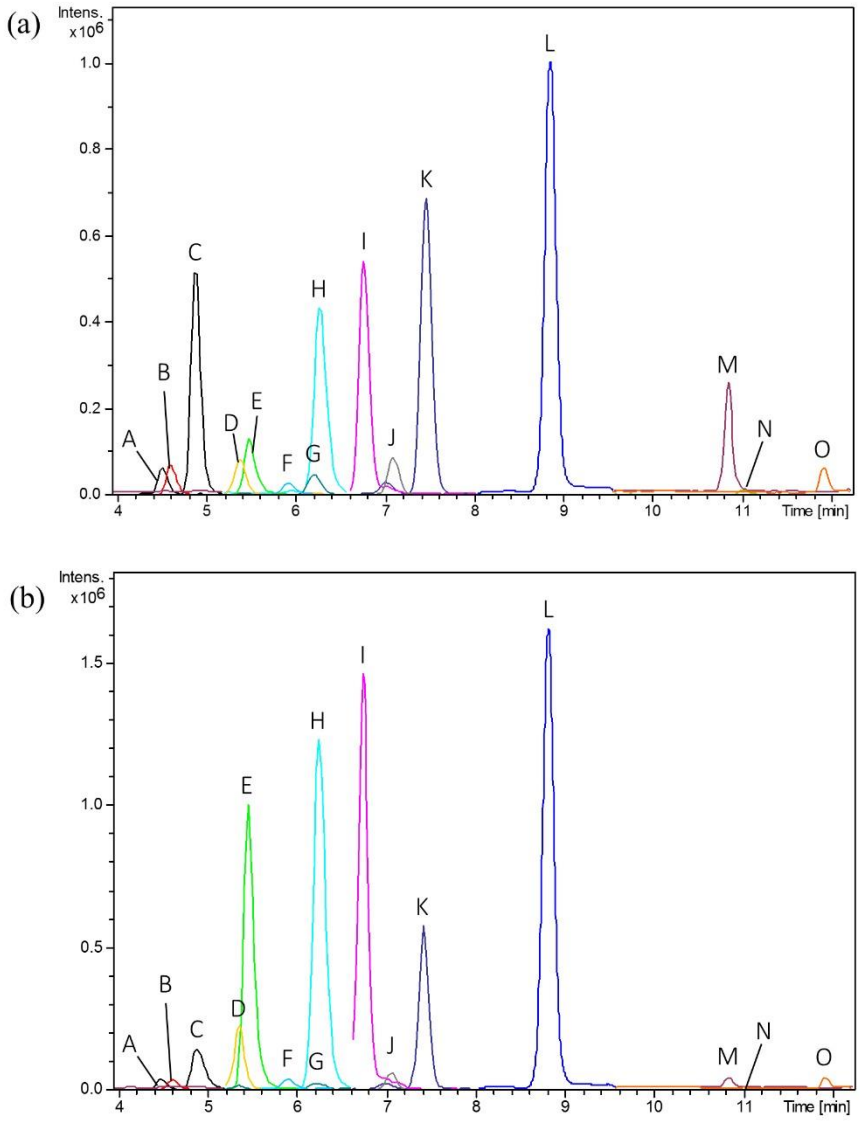


Figure 4.1. Annotated extracted ion chromatograms of a standard and a sample.

Extracted chromatograms for (a) standard with the dilution factor of 1/8 and (b) pFF from a control-fed pig with no blastocysts. Labels: A = myristic acid; B = α-linolenic acid; C = palmitoleic acid; D = arachidonic acid; E = linoleic acid; F = docosapentaenoic acid (DPA); G = dihomo-γ-linolenic acid (DGLA); H = palmitic acid; I = oleic acid; J = adrenic acid; K = margaric acid; L = stearic acid; M = arachidic acid; N = erucic acid; O = behenic acid.

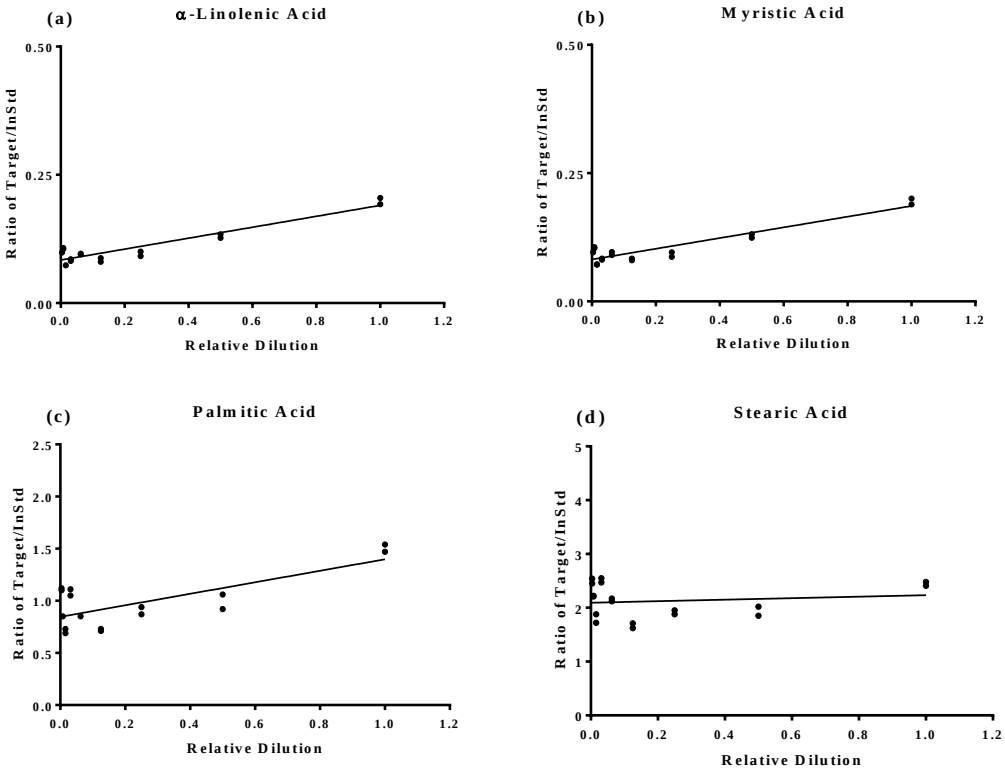


Figure 4.2. Standard curves from metabolomic analyses that showed contamination.

Standard curves for (a) α-linolenic acid, (b) myristic acid, (c) palmitic acid and (d) stearic acid, where the intercepts did not cross at zero due to contamination. Additionally, the solutions in the dilution series with the lowest concentrations also did not tend towards zero. The X-axes defined the relative dilution with one being the neat solution. The Y-axes defined the ratio of the area of the extracted ion chromatogram of the target fatty acid over the area of the chromatogram for the internal standard (InStd, margaric acid).

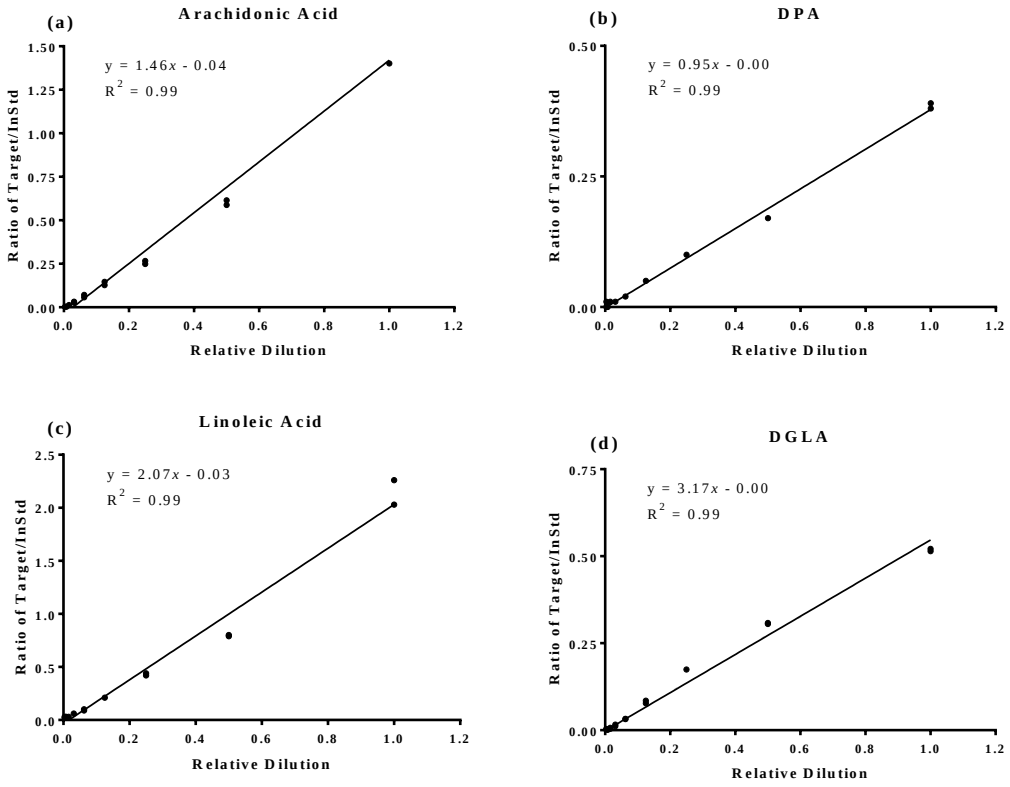


Figure 4.3. Linear standard curves from metabolomic analyses used to calculate concentrations.

Standard curves for (a) arachidonic acid, (b) docosapentaenoic acid (DPA), (c) linoleic acid and (d) dihomo-γ-linolenic acid (DGLA), all of which had intercepts close to zero and have linear curves with good R^2 values. The X-axes defined the relative dilution with 1 being the neat solution. The Y axes defined the ratio of the area of the extracted ion chromatogram of the target fatty acid over the area of the chromatogram for the internal standard (InStd, margaric acid).

Fatty acid	Inter-Assay CV (%)	Intra-Assay CV (%)
Arachidic	41.765	41.715
Palmitoleic	10.710	4.163
Oleic	25.300	22.735
Erucic	66.052	24.640
Linoleic	6.988	7.155
DGLA	26.040	5.690
Arachidonic	17.216	20.338
Adrenic	10.169	5.313
DPA	15.281	6.453

Table 4.4. Mean inter-assay and intra-assay coefficient of variations for each fatty acid.

The mean inter-assay coefficient of variation (CV) was determined by obtaining the inter-assay CV of each fatty acid in each sample (see **Appendices 4–5**) and finding the mean inter-assay CV for each fatty acid. The mean intra-assay CV was determined by calculating the intra-assay CV within each batch analysis and obtaining a mean from those four batches. Abbreviations: DGLA = dihomo-γ-linolenic acid; DPA = docosapentaenoic acid.

4.3.2. Concentrations of erucic acid and linoleic acid were different between control and high fibre samples

The nine fatty acids (mean concentration±standard error of the mean (SEM)) detected in this study in order of abundance were oleic acid (89.58±3.49 µg/mL), linoleic acid (85.15±4.94 µg/mL), arachidonic acid (51.31±3.77 µg/mL), adrenic acid (42.08±3.02 µg/mL), palmitoleic acid (26.67±1.45 µg/mL), DPA (16.10±2.29 µg/mL), DGLA (3.77±0.17 µg/mL), erucic acid (0.98±0.34 µg/mL) and arachidic acid (0.64±0.06 µg/mL). The fatty acid concentrations were analysed by either General Linear Model ANOVA or Kruscal Wallis which revealed a significant effect of diet on two of these fatty acids, erucic acid and linoleic acid ($p=0.024$ and $p=0.044$ respectively, **Figure 4.4a-b**). Two-sample t-tests confirmed that the pFF from high fibre-fed pigs had a higher mean±SEM concentration of erucic acid (0.18±0.01 µg/mL; 0.15±0.01 µg/mL) and a lower concentration of linoleic acid (75.67±6.21 µg/mL; 94.63±6.86 µg/mL) compared to control-fed pigs ($p=0.022$ and $p=0.053$ respectively, **Figure 4.4c-d**). The concentration of both of these fatty acids was not different between pFF of animals whose oocytes produced a blastocyst following IVF and pFF of animals whose oocytes did not produce blastocysts (**Figure 4.4e-f**). Additional Tukey comparison tests between samples from different diets and IVF outcomes identified the increased concentration of erucic acid in pFF of high fibre-fed pigs with no blastocysts compared to pFF control-fed pigs with no blastocysts and the decreased concentration of linoleic acid in pFF of high fibre-fed pigs with blastocysts compared to pFF control-fed pigs with blastocysts (**Figure 4.4a-b**). Oleic acid, arachidonic acid, adrenic acid, palmitoleic acid, DGLA, DPA and arachidic acid were not differentially expressed in the samples from different diets and IVF outcomes (**Table 4.5**). There were no interactions between diet and IVF outcome for any of the fatty acids measured, no significant effects from analysing the samples in different batches ($p>0.05$) for all fatty acid concentrations but arachidonic acid ($p=0.020$).

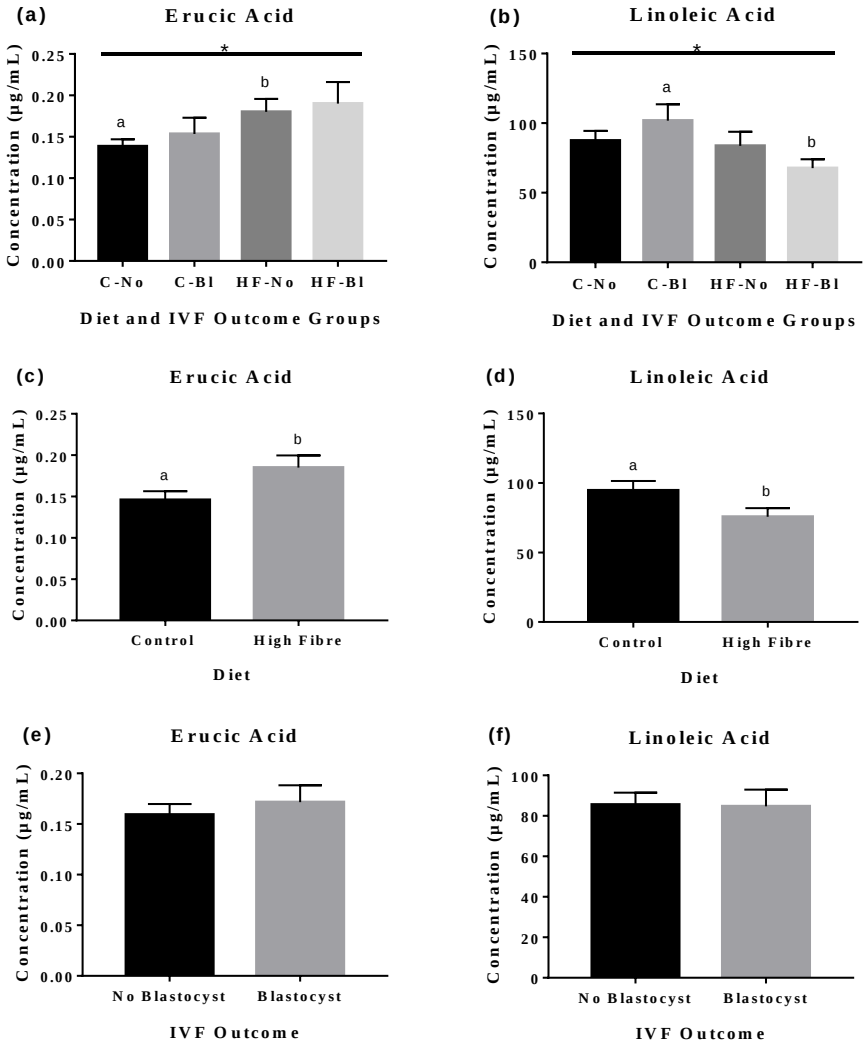


Figure 4.4. Concentration of erucic acid and linoleic acid in porcine follicular fluid from animals fed a control diet or a high fibre diet and associated with different *in vitro* fertilisation outcomes.

The concentration of (a) erucic acid and (b) linoleic acid in porcine follicular fluid (pFF) of animals associated with the different diets and different *in vitro* fertilisation (IVF) outcomes. Statistical significance was determined by *p≤0.05 from General Linear Model ANOVA and bars with superscripts “a” and “b” were statistically different between samples as determined by Tukey Comparison tests. The concentration of (c) erucic acid and (d) linoleic acid in pFF of animals associated with the different diets as well as the different IVF outcomes (e and f). Bars with superscripts “a” and “b” were statistically different between samples as determined by student t-tests. Abbreviations: All-C = all control group; All-HF = all high fibre group; C-No = control-fed animals without blastocyst; C-Bl = control-fed animals with blastocyst; HF-No = high fibre-fed animals without blastocyst; HF-Bl = high fibre-fed animals with blastocyst.

Fatty acid	**Mean Concentration (µg/mL)±SEM**				**P Values from ANOVA or Kruscal Wallis***		
	Control	*High Fibre*	*No Cleavage/ Blastocyst*	*Cleavage/ Blastocyst*	*Diet*	*IVF Outcome*	*Diet X IVF Outcome*
Arachidic	0.54±0.06	0.66±0.10	0.52±0.07	0.67±0.09	0.150	0.415	0.555
Palmitoleic	26.59±1.56	26.76±2.53	27.97±2.33	25.38±1.76	0.686*	0.453*	N/A
Oleic	87.90±4.55	91.26±5.46	90.24±4.80	88.93±5.28	0.633	0.859	0.378
Erucic	1.10±0.52	0.82±0.46	0.67±0.37	1.25±0.57	0.024	0.515	0.802
Linoleic	80.39±6.18	64.56±5.30	73.54±6.10	71.41±6.35	0.044	0.927	0.099
DGLA	3.67±0.23	3.86±0.24	3.71±0.26	3.83±0.21	0.571	0.729	0.117
Arachidonic	53.19±5.19	49.42±5.66	52.59±5.13	50.02±5.74	0.631	0.743	0.222
Adrenic	42.29±4.31	41.87±4.42	41.98±4.33	42.18±4.41	0.948	0.975	0.316
DPA	12.17±0.72	20.03±4.31	12.71±0.88	19.49±4.36	0.115	0.264	0.228

Table 4.5. Concentration of fatty acids in porcine follicular fluid from pigs fed a control or a high fibre diet and associated with different *in vitro* fertilisation outcomes.

The mean±standard error of the mean (SEM) porcine follicular fluid (pFF) concentrations of oleic acid, linoleic acid, arachidonic acid, adrenic acid, palmitoleic acid, docosapentaenoic acid (DPA), dihomo-γ-linolenic acid (DGLA), arachidic acid and erucic acid (µg/mL) in animals associated with the different diets and different IVF outcomes and analysed using General Linear Model ANOVA or Kruscal Wallis (*Nonparametric test) to identify any significant differences between samples from different regimens or associated with different IVF outcome or whether there were any interactions between the two factors.

4.3.3. Relationship between fatty acid concentrations with reproductive parameters

From an IVF study previously carried out by Dr Elizabeth M. Ferguson (Ashworth *et al.*, 2008), the oestradiol (E_2) concentrations, cleavage rates, blastocyst rates, blastocyst cell numbers, follicle sizes and pFF volumes associated with each pFF sample used in this study were recorded (**Appendix 2**). Regression analyses revealed no relationship between the fatty acid concentrations and each of these fertility parameters (**Table 4.6**). Arachidonic acid and DGLA pFF concentration decreased with follicle size ($p \leq 0.05$) but the R^2 values for each of these analyses were low ($R^2 \leq 0.2$) indicating that the relationship was not strong (**Table 4.7**). Arachidonic acid and adrenic acid pFF concentration had a trend towards decreasing with pFF volume ($p \leq 0.1$) and DGLA decreased with pFF volume ($p \leq 0.05$) but again the R^2 values for each of these analyses were low (≤ 0.2) indicating that the relationship was also not strong (**Table 4.7**).

Fatty Acid	(a) E_2 Concentration (ng/mL)		(b) Cleavage Rate (%)		(c) Blastocyst Rate (%)		(d) Blastocyst Cell Number	
	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*
Arachidic	0.038	0.364	0.007	0.790	0.000	0.986	0.064	0.269
Oleic	0.013	0.600	0.001	0.925	0.000	0.954	0.005	0.759
Erucic	0.000	0.987	0.153	0.209	0.000	0.976	0.043	0.365
Linoleic	0.012	0.615	0.108	0.296	0.092	0.339	0.011	0.650
DGLA	0.014	0.577	0.113	0.285	0.056	0.459	0.070	0.246
Arachidonic	0.014	0.580	0.015	0.709	0.015	0.700	0.010	0.662
Adrenic	0.007	0.691	0.018	0.678	0.006	0.809	0.000	0.911
DPA	0.002	0.831	0.142	0.254	0.122	0.292	0.009	0.711

Table 4.6. Regression analyses between mean porcine follicular fluid fatty acid concentrations versus pFF oestradiol concentration, cleavage rate, blastocyst rate, blastocyst cell number.

Mean porcine follicular fluid (pFF) concentrations of oleic, linoleic, arachidonic, adrenic, docosapentaenoic acid (DPA), dihomo-γ-linolenic acid (DGLA), arachidic and erucic acids (μg/mL) compared with; (a) pFF E_2 concentration; (b) cleavage rates of *in vitro* fertilised (IVF) oocytes (percentage of oocytes that cleaved from the total that were fertilised); (c) blastocyst rates of IVF oocytes (percentage of embryos that formed blastocysts from the total fertilised oocytes); (d) number of cells in blastocysts. Measurements for oestradiol concentrations, IVF and the recording of cleavage rates, blastocyst rates and blastocyst cell numbers were carried out by Dr Elizabeth M. Ferguson.

Fatty Acid	(a) Follicle size (mm)		(b) PFF volume (µl)	
	R^2	*p*	R^2	*p*
Arachidic	0.010	0.651	0.069	0.215
Oleic	0.085	0.167	0.096	0.141
Erucic	0.000	0.978	0.102	0.128
Linoleic	0.068	0.217	0.011	0.629
DGLA	0.174	0.043	17.34	0.043
Arachidonic	0.170	0.045	0.134	0.079
Adrenic	0.091	0.152	0.141	0.070
DPA	0.055	0.283	0.077	0.200

Table 4.7. Regression analysis between mean porcine follicular fluid fatty acid concentrations versus follicle size and follicular fluid volume.

The mean porcine follicular fluid (pFF) concentration of oleic acid, linoleic acid, arachidonic acid, adrenic acid, palmitoleic acid, docosapentaenoic acid (DPA), dihomo-γ-linolenic acid (DGLA) and arachidic acid and erucic acid was compared with the (a) diameter of the follicles from the same animals as well as the (b) pFF volume from the same animals. Follicle sizes and pFF volumes were recorded by Dr Elizabeth M. Ferguson.

4.4. Discussion

In this study, the concentrations of nine fatty acids was measured in pFF of 12 gilts fed either a control diet or a high fibre diet. Within each dietary regimen, three of the animals had oocytes that produced a blastocyst following IVF and three animals had oocytes which did not.

4.4.1. The liquid chromatography tandem mass spectrometry system detected prominent polyunsaturated fatty acids but not saturated fatty acids due to contamination

The concentration of prominent SFAs such as palmitic acid, stearic acid and myristic acid and the PUFA α-linoleic acid could not be accurately measured due to the presence of contaminants. The only SFA measured was arachidic acid. Despite the use of new glassware and MS-grade reagents, trace amounts of fatty acids contaminants were detected in "extracted" MS-grade dH_2O (the last solution of the dilution series) which should have no signals from fatty acids should have been detected. Additionally. The concentrations of these fatty acids in the lowest dilutions did not tend towards zero, making the curve non-linear. Previous studies that had carried out similar targeted metabolomic analysis of FF have often used gas chromatography (GC)-mass spectrometry or GC-MS/MS, which have been able to measure these fatty acids. With further optimisation, these fatty acids would likely have been able to be detected by the LC system that was available and the accuracy could have also been improved to obtain better CV values.

The interest in measuring these SFAs lie with the fact that they are abundant in pFF, especially palmitic acid and stearic acid (Yao *et al.*, 1980; Khandoker *et al.*, 1997). Additionally, pigs fed a diet rich in ω3-fatty acids increased the pFF concentration of SFAs but decreased the concentration of PUFAs, resulting in a higher SFA:PUFA ratio in pFF and making SFAs sensitive to different nutritional regimens (Warzych *et al.*, 2011). Of the PUFAs detected, their combined concentration does not appear to differ between samples from pigs fed different diets or associated with different outcomes,

although this does not necessarily mean that the SFA: PUFA ratio in pFF would not change.

The most abundant fatty acids detected in this study were consistent with the results of previously published studies that analysed the pFF of large porcine follicles, not including the SFAs that were not detected (Yao *et al.*, 1980; Khandoker *et al.*, 1997). However, the concentrations detected in this study were slightly higher, with oleic acid being as much as three times more concentrated than the concentrations listed in Khandoker *et al.*, (1997). This is likely due to improved sensitivity of the detection techniques in the last 20 years.

4.4.2. There were no associations between fatty acid concentrations and different reproductive parameters

The concentrations of the fatty acids measured did not differ between pFF of pigs whose oocytes cleaved and produced a blastocyst and pFF whose oocytes did not cleave or produce a blastocyst following IVF. This agreed with the bovine studies of Sinclair, (2008) and Aardema *et al.*, (2013, 2015), where cleavage and blastocyst rates were independent of increased fatty acid concentrations.

In agreement with Leroy *et al.*, (2004) there were no effects of follicle size and pFF volume, though this was somewhat expected as the samples were taken from a follicle population of similar sizes. However, relationships between fatty acids and follicle size have been observed, with linoleic acid content in bovine FF decreasing with increasing follicle size (Homa and Brown, 1992; Zachut *et al.*, 2008). Additionally, stearic acid and palmitic acid were present at higher concentrations in prepubertal gilts compared to cyclic gilts (Pawlak *et al.*, 2012). This implies that fatty acid concentrations in pFF tend to decrease with more mature follicles. Whilst there were no associations with linoleic acid and follicle size (likely due to the uniformity in follicle size), the lowering of linoleic acid in high fibre pFF may indicate that the follicles from high fibre pigs were more mature as the follicles form control pigs. This is contrary to the discussions in chapter 3, sections 3.4.2 and 3.4.3. Additionally, the lower fatty acid concentrations in mature follicles could potentially have implications

for IVF as most IVF experiments use abattoir derived ovaries from pre-pubertal gilts and the maturation of oocytes with pre-pubertal pFF rich in fatty acids may not be optimal.

There were no associations between the concentrations of the fatty acids detected with FF oestradiol (E_2) concentration. There were also no measurements for FF progesterone (P_4) concentration which could be used to obtain an E_2:P_4 ratio; a ratio greater than one being indicative of active follicles. Measurements for pFF P_4 concentration could have been carried out in this study, were it not for the limitations of time and resources. It would have been interesting to asses this as in bovine studies, the E_2:P_4 ratio in FF negatively correlated with free fatty acid levels (Renaville *et al.*, 2010; Aardema *et al.*, 2015). The E_2:P_4 ratio is also subject to differences in diet as pregnant and lactating cows and sheep fed a diet with high levels of either oleic, linoleic and conjugated acids had increased FF E_2 and P_4 concentrations and E_2:P_4 ratios compared to their control counterparts (Castañeda-Gutiérrez *et al.*, 2007; Zachut *et al.*, 2008; Wonnacott *et al.*, 2010).

Zachut *et al.*, (2008) suggested that the way in which the changes in E_2 and P_4 levels occurred was through cholesterol dependent steroidogensis as they observed that cows fed a diet supplemented with high levels of oleic and linoleic acids had an increase in P450 aromatase expression in granulosa cells (Zachut *et al.*, 2008). However, the way in which the increased fatty acids act on the hormonal levels of FF remain uncertain as when granulosa cells were cultured in the presence of high-density lipoproteins and fatty acids, there were no effects on E_2 or P_4 production (Jorritsma *et al.*, 2004; Wonnacott *et al.*, 2010). Alternatively, Hughes *et al.*, (2011) suggested that ω3-fatty acids increased P_4 production solely in theca cells, which was also associated with an increase in steroidogenic acute regulatory protein transcript expression.

4.4.3. The concentration of erucic acid and linoleic acid were different between follicular fluids of control and high fibre-fed pigs

The targeted metabolic analysis revealed the increased concentration of erucic acid and the decreased concentration of linoleic acid in pFF of high fibre pigs. However,

the CV values for the erucic acid make this result lass reliable. However, the measurements for linoleic acid were among the most reliable with good inter- and intra-assay CV values. Both fatty acids can be provided through nutrition, erucic acid being rich in rapeseed oil and linoleic acid being an essential fatty acid in humans and also prevalent in many plant based oils.

Rapeseed meal is a good source for erucic acid and when included into the diets of pregnant pigs, piglet growth improved, though there were no effects on overall reproductive performance of gilts (Opalka *et al.*, 2001; Hanczakowska and Świątkiewicz, 2013). However, the direct benefits of erucic acid alone on oocyte maturation, have not been examined. This is likely due to the low levels of the fatty acid in the FF of several species.

Linoleic acid is also a fatty acid that is provided in the diet, and as described in chapter 1, section 1.3.2, its main function is to act as a precursor to arachidonic acid in the synthesis of prostaglandins but is also involved in the formation of cell membranes. However, unlike erucic acid, it has been associated with several reproductive processes. In humans, linoleic acid appears to be beneficial since its levels increased in hFF of pregnant women compared to non-pregnant women and were positively correlated with fertilisation rate, whilst levels of arachidonic acid were negatively correlated (Shaaker *et al.*, 2012). However, these beneficial observations are contradictory as levels of arachidonic and linoleic acid derivatives were higher in hFF associated with oocytes that did not show two pronuclei or underwent degeneration (Ciepiela *et al.*, 2015). Additionally, linoleic acid co-culture with bovine oocytes significantly inhibited the breakdown of germinal vesicles in a dose-dependent manner and preovulatory follicle diameter was greater for cows fed a diet supplemented with linoleic acids compared to cows fed a diet (Homa and Brown, 1992; Zachut *et al.*, 2008). However, even with bovine studies, the negative effects of linoleic acid are multifaceted as lactating dairy cows fed a diet supplemented with linoleic acid had increased plasma prostaglandin F2α concentration and increased pregnancy rates following insemination compared to control fed cows (Juchem *et al.*, 2010).

4.4.4. Soyabean oil and fishmeal oil are potential sources of linoleic acid in the control and high fibre diets

The composition of the control and high fibre diets are summarised in **Appendix 3**. Most notable differences included the increase of unmolassed sugar beet pulp by 50% and the 45% decrease in barley in the high fibre diet. However, there is also a decrease by 1.5%, 0.36% and 0.13% in hipro soya, fishmeal and soya oil respectively. These were all small changes in these constituents, although there could have been a combined effect of reducing them as a whole.

The decrease in linoleic acid was likely due to lower levels of Hippro soya and/or soya oil as soya bean is known to be rich in linoleic acid (Ponter *et al.*, 2012). Additionally, rats and cows given a diet of soya isoflavones or soya bean showed increased linoleic acid in their FF and plasma respectively (Ponter *et al.*, 2012; Wang *et al.*, 2013b).

Evidence towards the changes in pFF linoleic acid as a result of differences in soya extracts is provided by a nutritional study whereby gilts were fed either a control diet or an experimental diet rich in ω3-fatty acids (Warzych *et al.*, 2011). The barley and SBP content in the experimental diet in Warzych *et al.*, (2011) was comparable to the control diet used in this study (Ashworth *et al.*, 2008). However, Warzych *et al.*, (2011) also observed a decrease in linoleic acid concentration in pFF of pigs fed the experimental diet, implying that the alteration of linoleic acid in pFF was not directly due to the barley and/or sugar beet pulp content. The similarities between the experimental diet in Warzych *et al.*, (2011) and the high fibre diet in Ashworth *et al.*, (2008) are the reduced content of soybean meal and rapeseed meal in the former and hipro soya, soya oil and fishmeal oil in the latter. However, Warzych *et al.*, (2011) also reported no significant change in linoleic acid content between the two diets, suggesting that the different compositions in diet are implicated in the digestion, metabolism and transport of the fatty acids and not necessarily the fatty acid content of the diet.

There have also been studies that have assessed the effects of a linoleic acid rich diet on the rumen system. Interestingly, heifers offered a diet supplemented with fish oil

had decreased FF concentrations of linoleic acid but increased concentration of *cis*-9, *trans*-11 CLA and unchanged concentrations *trans*-10, *cis*-12 CLA (Childs *et al.*, 2008). This difference observed in the concentrations of linoleic acid and its conjugated isomers is intriguing as their effects on reproduction appear to also differ, as described in chapter 5, section 5.1.1.

4.4.5. Conclusion and next steps

The targeted metabolomic analysis described in this chapter has measured the concentration of nine fatty acids in pFF from 12 animals, six of which were fed a control barley-based diet, the other six a high fibre diet supplemented with unmolassed sugar beet pulp. Four of the most abundant fatty acids (myristic, palmitic, stearic and α-linolenic) were not able to be detected with the LC-MS/MS system unlike other studies that utilised a GC system. There were no effects of the diet on the concentration of seven of the nine fatty acids acid measured. There were also no associations of any of the fatty acid concentrations with follicle size, pFF volume, pFF E_2 concentration, cleavage rate, blastocyst formation or blastocyst rate. However, the study identified the increased concentration of erucic acid and the reduced concentration of linoleic acid in pFF of high fibre-fed gilts. Linoleic acid has previously been implicated in detrimental effects of oocyte maturation, especially in bovine studies, but its effect in pigs has yet to be determined. Therefore, as described in chapter 5 porcine oocytes were incubated in different concentrations of linoleic acid to see if oocyte IVM was affected in any way.

5. The Effect of Linoleic Acid on *In Vitro* Maturation of Porcine Oocytes

5.1. Introduction

The targeted metabolomic analysis of porcine follicular fluid (pFF) described in chapter 3 identified that the concentration of linoleic acid was lower in pFF of pigs fed the high fibre diet compared to the pFF of pigs fed the control diet. However, the potential downstream effects on porcine oocyte maturation as a result of a lower pFF concentration of linoleic acid remain uncertain. The majority of published studies that have assessed the effects of elevated linoleic acid in the microenvironment of maturing oocytes have been carried out on bovine cumulus-oocyte-complexes (COCs). The studies that have used porcine COCs have only assessed the effects of conjugated linoleic acid (CLA) (Prates *et al.*, 2013a, b; Jia *et al.*, 2014). Therefore, in this final results chapter, the effects of linoleic acid on oocyte *in vitro* maturation (IVM) were examined.

5.1.1. Linoleic acid and conjugated linoleic acid supplementation into *in vitro* maturation systems

Linoleic acid is a polyunsaturated ω6-fatty acid composed of 18 carbon atoms with two double bonds, both with *trans*- orientation, on the ninth and twelfth carbon atoms of the hydrocarbon chain. CLA refers to the 28 positional and geometrical isomers of linoleic acid, but only two of the isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, have been shown to be biologically active (Banni, 2002). However, the notable differences in the position and orientation of the double bonds between the three molecules (**Figure 5.1**) appear to have different effects on oocyte maturation, fertilisation and subsequent embryo development *in vitro*, as described below.

Trans-10, cis-12 CLA

In pigs, no significant differences were observed in the percentage of MII oocytes following IVM in North Carolina State University Medium 23 with and without 100

µM *trans*-10, *cis*-12 CLA (Prates *et al.*, 2013a, b). However, there is evidence that suggests that the supplementation of *trans*-10, *cis*-12 CLA had an optimum concentration of 50 µM; Jia *et al.*, (2014) matured porcine COCs in either TCM 199 based IVM medium or the same medium supplemented with 10, 25, 50 or 100 µM *trans*-10, *cis*-12 CLA for 44 hours. The percentage of MII oocytes was significantly higher when matured with 50 µM CLA compared to the normal TCM 199 medium and TCM 199 supplemented with 100 µM CLA but did not significantly differ from TCM 199 formulations supplemented with 10 µM and 25 µM CLA (Jia *et al.*, 2014). Additionally, when maturation was monitored after 22 and 36 hours, the percentage of oocytes at metaphase I (MI) at 22 hours and the percentage of metaphase II (MII) oocytes at 36 hours were significantly higher with 50 µM CLA compared to normal TCM 199 medium (Jia *et al.*, 2014). The percentage of cleaved oocytes increased along with the blastocyst rate of oocytes that were matured in 50 µM *trans*-10, *cis*-12 CLA, following parthenogenic activation (PA) of oocytes and culture in porcine zygote medium for seven days (Jia *et al.*, 2014).

The effects of supplementing TCM 199 medium with *trans*-10, *cis*-12 CLA on bovine IVM differ slightly from that of porcine IVM; maturation with various concentrations (15, 25, 50 and 100 µM) of *trans*-10, *cis*-12 CLA did not affect bovine oocyte maturation (Lapa *et al.*, 2011; Absalón-Medina *et al.*, 2014). However, with a very high concentration (200 µM) of *trans*-10, *cis*-12 CLA, which has not been tested in pigs, maturation rate was significantly decreased (Absalón-Medina *et al.*, 2014). Additionally, an increase in cleavage rate was observed with 15 and 50 µM *trans*-10, *cis*-12 CLA whilst a decrease in blastocyst rate was observed with 50, 100 and 200 µM following PA (Absalón-Medina *et al.*, 2014). However, there was no effect of 100 µM *trans*-10, *cis*-12 CLA on either cleavage or blastocyst rate following *in vitro* fertilisation (IVF) (Lapa *et al.*, 2011).

Cis-9, trans-11 CLA

As with the *trans*-10, *cis*-12 isomer, supplementing TCM 199 medium with 15, 25, 50 or 100 µM of *cis*-9, *trans*-11 CLA did not affect bovine oocyte maturation rates, although this was also the case with 200 µM of *cis*-9, *trans*-11 CLA (Absalón-Medina

et al., 2014). Additionally, as with *trans*-10, *cis*-12 CLA, 15 µM *cis*-9, *trans*-11 CLA increased cleavage rates and 100 µM and 200 µM CLA decreased blastocyst rates following PA (Absalón-Medina *et al.*, 2014).

Linoleic acid

Despite being the most abundant fatty acid in bovine follicular fluid (Bender *et al.*, 2010), supplementing linoleic acid to bovine IVM medium produced consistently negative effects. Supplementation of TCM 199 medium with 50, 100 and 200 µM linoleic acid decreased the proportion of fully expanded COCs, increased the proportion of COCs that did not expand, decreased the percentage of MII oocytes and increased the percentage of oocytes arrested at MI and GV after 22–24 hours of IVM (Marei *et al.*, 2010; Carro *et al.*, 2013; Khalil *et al.*, 2013). Additionally, oocytes matured in TCM 199 with 100 µM linoleic acid had significantly lower blastocyst rates compared to oocytes matured in normal medium, whilst the cleavage rates were either unchanged or lower with linoleic acid (Marei *et al.*, 2010; Khalil *et al.*, 2013).

As with the CLA isomers, supplementation of IVM medium with linoleic acid gave different results between different species. The number of ovine MII oocytes significantly increased with TCM 199 supplemented with 50 and 100 µM linoleic acid compared to TCM 199 medium alone (Amini *et al.*, 2016). Additionally, the expansion of cumulus cells and cleavage rates were significantly decreased when oocytes were matured in TCM 199 with 200 µM linoleic acid, whilst blastocyst rate was significantly lower with all of the linoleic acid concentrations (Amini *et al.*, 2016). In goats, IVM medium with 200 µM linoleic acid an had increased number of unfertilised oocytes and rate of polyspermy following IVF as well as decreased blastocyst rate after nine days of embryo culture (Roura *et al.*, 2017). However, the number of cells in the inner cell mass of blastocysts produced from PA was significantly higher with the supplementation of linoleic acid (Roura *et al.*, 2017).

In summary, there have been no IVM studies carried out on porcine COCs to test the effects of elevated concentrations of linoleic acid on oocyte maturation. The only porcine IVM studies that have been carried out have assessed the effects of *trans*-10,

cis-12 CLA. The effects of this isomer differ between porcine IVM and bovine IVM studies. Additionally, within the bovine studies, there were differences observed between the effects of *trans*-10, *cis*-12 CLA and the effects of *cis*-9, *trans*-11 CLA and linoleic acid. Furthermore, the effects of linoleic acid were not entirely consistent between different species (bovine, ovine and caprine). Therefore, the potential effect of linoleic acid on porcine oocyte maturation is yet to be determined.

5.1.2. Hypothesis and experimental aims

The hypothesis was that increased linoleic acid in the microenvironment of a maturing oocyte would be detrimental to the development of that oocyte. The rationale being that the results of the metabolomic analysis revealed a lower concentration of linoleic acid in pFF of high fibre-fed pigs. These pigs were also associated with an increased proportion of MII oocytes, following IVM and *in vivo* maturation in the ovary (Ferguson *et al.*, 2007; Weaver *et al.*, 2013, respectively).

To test the hypothesis, IVM experiments were carried out whereby COCs were matured in maturation medium supplemented with different concentrations (0, 50, 100 and 200 µM) of linoleic acid. These experiments were carried out to identify whether increased linoleic acid affected key indicators of good maturation such as cumulus expansion and the proportion of MII oocytes.

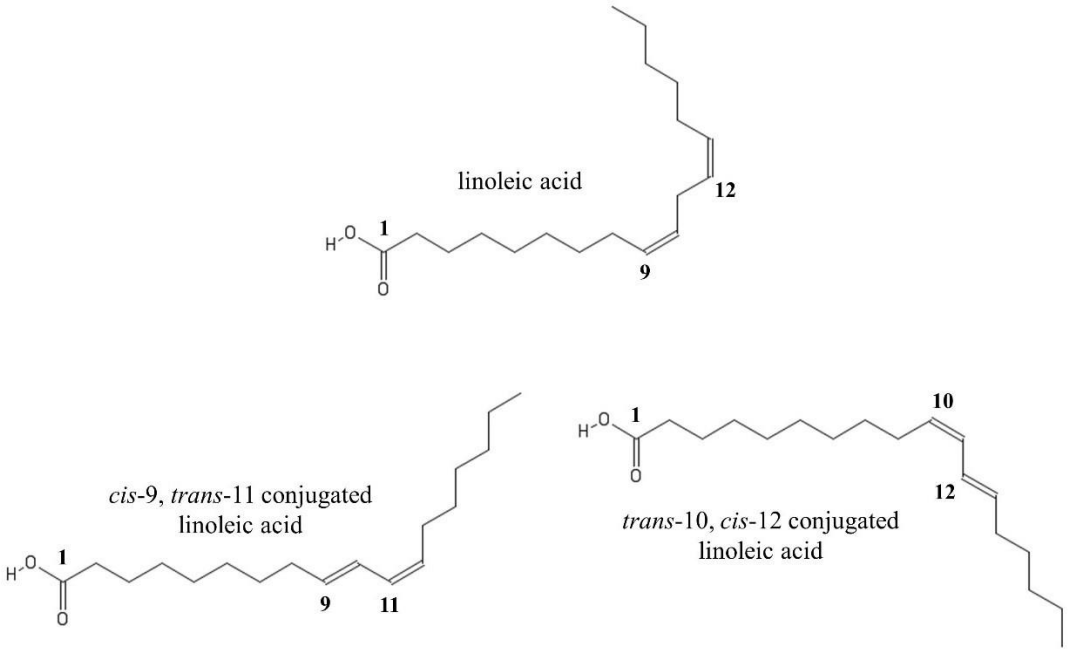


Figure 5.1. Chemical structures of linoleic acid, *cis*-9, *trans*-11 conjugated linoleic acid and *trans*-10, *cis*-12 conjugated linoleic acid.

Images were created using PubChem Sketcher V2.4 which was accessed on 30/08/2017. The labelled carbon atoms on the hydrocarbon chain include the first carbon atom that has the carboxylic acid functional group and the carbon atoms in which the double bonds appear.

5.2. Materials and Methods

5.2.1. Preparation of stock reagents

All chemicals and equipment were supplied by Sigma Aldrich unless specified.

Glucose (305 mM)

D-glucose (0.549 g) was dissolved in 10 mL deionised water (dH_2O), separated into 210 µl aliquots and stored at -20 °C.

Cysteine (57 mM)

L-cysteine (0.069 g) was dissolved in 10 mL dH_2O, separated into 210 µl aliquots and stored at -20 °C.

Luteinising hormone (1 mg/mL)

Luteinising hormone (LH, 0.01 g) was dissolved in 10 mL dH_2O, separated into 15 µl aliquots and stored at -20 °C.

Follicle stimulating hormone (10 mg/mL)

A vial of follicle stimulating hormone (FSH, 50 mg) was dissolved in 10 mL dH_2O, separated into 5 µl aliquots and stored at -20 °C.

Epidermal growth factor (1 mg/mL)

A vial of epidermal growth factor (EGF, 1 mg) was dissolved in 1 mL dH_2O, separated into 5 µl aliquots and stored at -20 °C.

Penicillin (15 mg/mL)

Penicillin (0.15 g) was dissolved in 10 mL dH_2O, separated into 110 µl aliquots and stored at -20 °C.

Streptomycin (10 mg/mL)

Streptomycin sulphate (0.1 g) was dissolved in 10 mL dH_2O, separated into 110 µl aliquots and stored at -20 °C.

Linoleic acid (2 mg/mL)

Linoleic acid (10 mg) was dissolved in 5 mL TCM 199, separated into 200 µl aliquots and stored at -20 °C.

Phosphate buffered saline

Phosphate buffered saline (PBS) tablets (Thermo Fisher Scientific) were dissolved in 100 mL dH_2O per tablet. The pH of the buffer was adjusted to pH 7.4 using 12 M hydrochloric acid (HCl) and/or 5 M sodium hydroxide (NaOH). Each tablet was composed of 8 g/L sodium chloride (NaCl), 0.2g/L potassium chloride (KCl), 1.15g/L potassium phosphate (KH_2PO_4) and 0.24 g/L sodium phosphate (Na_2HPO_4).

Hyaluronidase (1320 units/mg)

PBS (8.8 mL) was added to 0.1 g of hyaluronidase to give a concentration of 0.15 units/mL (or 0.0114 g/mL) which was separated into 20 µl aliquots and stored at -20 °C.

0.5% Triton X-100

Triton X-100 (5 mL) was dissolved in 995 mL of sterile PBS to make 1 L and stored at 4 °C.

0.05% Tween-20

Tween-20 (0.5 mL, VWR) was dissolved in 999.5 mL of sterile PBS to make 1 L and stored at 4 °C.

2% Bovine serum albumin

Bovine serum albumin (BSA) (20 g, Thermo Fisher Scientific) was dissolved in 1 L of sterile PBS and stored at 4 °C.

5.2.2. Preparation of porcine follicular fluid

Prior to carrying out IVM experiments with linoleic acid supplementation, trial IVM experiments were carried out to confirm that the protocol was suitable and that all the equipment and resources were available. From these trial IVM experiments, spare pFF had been collected from 112 abattoir derived ovaries; the identities of the animals the ovaries had been taken from was uncertain, but unhealthy or premature ovaries were discarded. Therefore, the ovaries that were used could not be paired with each other. After the IVM runs, the pFF collected was centrifuged for 30 min at 3,000 g. The supernatant was removed, and filter sterilised through a Ministart single filter 0.2 mm (Thermo Fisher Scientific). The pFF was then divided into 1.01 mL aliquots and stored at -20 °C.

A stock of experimental pFF was then prepared, which was used to supplement the maturation media. It was composed of 21 mL of the spare abattoir derived pFF collected from the trial IVM experiments. In addition to this, 200 µl of "Pool 2" pFF (see section 2.2.1 in chapter 2) was taken from 39 animals (see **Appendix I**) to make a total volume of 28.8 mL; the "Pool 2" pFF had been collected by Dr Elizabeth Ferguson for the study described in Ashworth *et al.*, (2008). Therefore, the stock of experimental pFF was composed of pFF from pre-pubertal gilts and post-pubertal gilts, the former at various stages of their oestrous cycle.

5.2.3. Preparation of media

All chemicals were supplied by Sigma Aldrich unless specified.

TL HEPES PVA (Porcine Handling Medium)

A 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) stock solution was made that would last throughout the whole experiment. For 500 mL stock solution,

3.332 g NaCl, 0.120 g KCL, 0.084 g sodium bicarbonate, 0.020 g NaH_2PO_4, 0.050 g magnesium chloride, 1.192 g HEPES, 0.450 g D-Glucose and 0.030 g Penicillin G were dissolved in 450 mL dH_2O, mixing after the addition of each chemical. The stock solution was filter sterilised with a Ministart single filter (0.2 µm) and stored at 4 °C for up to 3 months.

A 200 mM sodium pyruvate solution was made for every new batch of medium; 0.1004 mg sodium pyruvate was dissolved in 5 mL dH_2O. For a 50 mL solution of the TL HEPES PVA medium, 25 µl gentamicin (50 mg/mL stock), 0.0148 g calcium chloride and 0.005 g polyvinylalcohol 93 µl sodium lactate and 50 µl of the sodium pyruvate solution were dissolved in/added to 45 mL HEPES stock solution, mixing after the addition of each chemical. The pH and osmolality of the medium were adjusted to 7.4±0.05 (with 12 M HCl and/or 5 M NaOH) and 260–280 mOsm/kg respectively. The medium was filter sterilised with a Ministart single filter (0.2 µm) and stored at 4 °C for up to 2 weeks.

Tissue Culture Medium 199 maturation medium

A 91 mM sodium pyruvate solution was made for every new medium; 98 mg sodium pyruvate was dissolved in 10 mL dH_2O. To make the maturation medium, 200 µl of the sodium pyruvate solution was added to 20 mL of Tissue Culture Medium 199 (TCM 199, with no phenol red), along with 200 µl of 305 mM glucose, 10 µl of 1 mg/mL LH, 1 µl of 10 mg/mL FSH, 200 µl of 57 mM cysteine, 2 µl of 1 mg/mL EGF, 100 µl of 15 mg/mL penicillin, 100 µl of 10 mg/mL streptomycin and 2 mL filtered experimental pFF.

Maturation medium supplemented with linoleic acid

The required final concentrations of linoleic acid in TCM 199 were 0, 50, 100 and 200 µM, which equal to 0 mg/mL, 0.014 mg/mL, 0.028 mg/mL and 0.056 mg/mL respectively. A 200 µl aliquot of 2 mg/mL linoleic acid was taken from -20 °C and was used to make 1 mg/mL and 0.5 mg/mL solutions of linoleic acid, where TCM 199 was the diluent.

To make the four different media, the 20 mL TCM 199 solution was split into 3 x 3 mL aliquots and one 11 mL aliquot. To one of the 3 mL aliquots, 84 µl of 2 mg/mL linoleic acid was added to make 200 µM linoleic acid. To the second 3 mL aliquot, 84 µl of 1 mg/mL linoleic acid was added to make 100 µM linoleic acid. To the third 3 mL aliquot, 84 µl of 0.5 mg/mL linoleic acid to make 50 µM linoleic acid. The 11 mL aliquot had no additional linoleic acid and was the "control" but did have 308 µl of TCM 199 to keep the osmolality changes between the different media consistent.

The pH and osmolality of each medium were adjusted to 7.3–7.4 (with 12 M HCl and/or 5 M NaOH) and 280±5 mOsm/kg respectively. The medium was filter sterilised through a Ministart single filter 0.2 mm and stored at 4 °C for up to 2 weeks.

5.2.4. Preparation for oocyte aspiration and *in vitro* maturation

Several steps were carried out to ensure that the oocytes would remain under warm conditions throughout the protocol. A 90 mm petri dish and two 500 mL bottles of sterile PBS were stored in a 39 °C, 5% carbon dioxide (CO_2) incubator for at least 12 hours before aspiration.

Prior to maturation, 400 µl of maturation medium was added to each well of a Nunc four well plate (Thermo Fisher Scientific); one well containing the control medium, the other three containing a medium supplemented with 50, 100 or 200 µM of linoleic acid. Additionally, 1.5 mL of control maturation medium was added to two Nunc 35 mm dishes (Thermo Fisher Scientific), 2 mL of TL HEPES PVA was added to three 35 mm dishes and 10 mL of TL HEPES PVA was added to a 30 mL universal tube. The plate, dishes and universal tube were placed in the 39 °C, 5% CO_2 incubator for at least 1 hour before they were used.

5.2.5. Collection and transport of pig ovaries

Pig ovaries were collected from the local abattoir (Foundry Road, Shotts, ML7 5DX, UK) and transported back in a heat insulating flask (Argos, 11–15 North Bridge, Edinburgh, EH1 1SB, UK) containing PBS at 37 °C. Collection and transport of ovaries was carried out by Adrian Ritchie (Dryden Farm). The ovaries were usually

collected at approximately 7 am and were not kept in the flask longer than 5 hours. Upon arrival, the temperature of the PBS was measured with a thermometer to check that it had remained at 37 °C after the journey from abattoir.

5.2.6. Porcine ovary aspiration

Once the ovaries arrived and had their temperatures recorded, the warm PBS was taken from the 39 °C incubator. The ovaries were poured gently into a colander (IKEA, Main Street, Loanhead, Straiton, Edinburgh, EH20 9PW, UK), washed with 750 mL of the pre-warmed PBS and transferred into an insulated tub (IKEA) containing the remaining 250 mL PBS. The lid was kept on the tub as much as possible during the aspiration. In the aspiration hood, ovaries were taken one by one from the tub for aspiration. Ovarian follicles larger than 3 mm in size were aspirated using an 18-gauge needle from BD Microlance (Thermo Fisher Scientific) and a 10 mL syringe (Terumo, 2101 Cottontail Lane, Somerset, New Jersey, 08873, USA). The needlepoint was inserted downward into the ovary 2–3 mm away from chosen follicle to penetrate the follicle from underside. With the needle in the follicle, the syringe plunger was slowly withdrawn to remove the follicular contents, avoiding the aspiration of air which could damage the cumulus cells surrounding the oocyte. After every 2–4 ovaries, the needle was removed from the syringe and the fluid was collected into one of the universals from the water bath which was then placed back in the water bath. This procedure was repeated until all the follicles of appropriate size were aspirated. Unhealthy, cystic or immature ovaries were rejected; unhealthy ovaries included cystic ovaries or ovaries with discolouration (bruising) whilst immature ovaries only had follicles smaller than 3 mm. The number of ovaries aspirated, and the number of ovaries rejected were recorded. When all the suitable ovaries had been aspirated, the universal tube was left to stand upright for 5 minutes in the water bath and 5 minutes in the hood to allow the contents (cells) to collect at the bottom. Excess follicular fluid (FF) was transferred to the other universal tube and placed back in the water bath, leaving settled oocytes and cumulus cells at the bottom of the tube. The universal tube with pFF was kept in the water bath until the end of the IVM protocol and was then processed as described

previously. The universal with the cells was wiped down with 70% ethanol and placed in the culture hood.

5.2.7. Oocyte search and maturation

The universal tube containing the oocytes and cumulus cells was rinsed with 10 mL of the pre-warmed TL HEPES PVA and poured into the warm 90 mm petri dish. COCs were visualised on the Wild M3Z Herbrugg stereomicroscope (Leica, Balliol Business Park, Newcastle upon Tyne, NE12 8EW, UK), and using a p20 pipette they were transferred to one warm 35 mm dish of TL HEPES PVA. The total number of COCs found was recorded. Good quality oocytes, with full/even cytoplasm and two to three layers of cumulus cells were selected using a p20 pipette and transferred to another warm 35 mm dish of TL HEPES PVA; oocytes with cracked zona pellucida or no cumulus cells were avoided. The selected COCs were washed through the other 35 mm dish of TL HEPES PVA and the two 35 mm dishes of maturation medium. The COCs were placed into the wells of the pre-warmed four well plate; 30–39 COCs were incubated with 400 µL of medium per well, 20–29 COCs in 300 µL of medium or 1519 COCs in 200 µL of medium. The numbers of COCs matured varied, depending on the number of good quality COCs. The allocation of treatments to each well within the 4-well plate changed between experiments so that each medium would not be in the same well for all experiments. The total number of COCs matured in each well was recorded. The COCs were incubated for 44 hours at 39 °C, 5% CO_2. Aliquots (1 mL) of each medium (before maturation) was stored at -80 °C.

5.2.8. Imaging the cumulus-oocyte-complexes

After the 44-hour incubation, the plate was taken from the 39 °C, 5% CO_2 incubator and placed on the Zeiss Axiovert 25 inverted fluorescence microscope (Zeiss, 509 Coldhams Lane, Cambridge, CB1 3JS, UK). Bright field images of all the COCs in each well were taken (at x5 magnification) using the microscope and Zen Blue software (Zeiss). The images were converted into TIF files. Scale bars (200 µm) were added to the TIF files using Zen Blue software.

5.2.9. Fixing and staining oocytes for assessment of maturation

The methodology described is based on the protocol by Prentice-Biensch *et al.*, (2012). Using a p200 pipette, each well was mixed to remove the cumulus cells from the oocytes. The oocytes were transferred to a new four well plate containing 400 µL of TL HEPES PVA for washing and mixing again. The media after maturation were collected and stored at -80 °C. The TL HEPES PVA was removed and 400 µL TL HEPES PVA containing 0.15 units/mL hyaluronidase was added to each well. The oocytes were incubated in this solution for 5 minutes and mixed again to remove remaining cumulus, before the solution was removed and replaced with 400 µL TL HEPES PVA for washing. This TL HEPES PVA was also removed and the oocytes in each well were fixed in 400 µL 4% paraformaldehyde (PFA) for 30 minutes at room temperature on a stirrer. The zona pellucida of every one of the fixed oocytes was perforated with 0.5% Triton X-100 and 0.05% Tween-20 for 30 minutes each then blocked with 2% BSA for 1 hour. Perforation of the oocytes would allow the staining to be more effective. The oocytes were incubated overnight at 4 °C in 1:500 anti-lamin A+C (Abcam) made up in 2% BSA. The following day, the oocytes were stained with secondary antibody solution, 1:1000 Alexa Fluor 488 labelled Goat anti-rabbit IgG (Abcam) for 1 hour, followed by another 1-hour incubation in 2 µg/mL 4',6-diamidino2-phenylindole (DAPI, Sigma Aldrich). The oocytes were kept in the same order of wells throughout the fixing and staining procedure. After each incubation (in PFA, Triton X-100, Tween-20, BSA and anti-lamin), the oocytes in each well were washed in 400 µL pre-prepared Dulbubecco's PBS (VWR) three times. The final wash after DAPI staining was 3 x 400 µl dH_2O, after which the oocytes were transferred to separate wells of microscope slides with reaction wells (Marienfeld, Am Wöllerspfad 4, 97922 Lauda-Königshofen, Germany) and fixed with a drop of Prolong Diamond Antifade Mountant (Molecular Probes, Thermo Fisher Scientific) and covered with a cover slip (VWR). The slides were stored in the dark (in foil covered-drying rack) in the cold room until imaging.

5.2.10. Fluorescent imaging of DAPI and anti-lamin stained oocytes

The slides were taken from the cold room and fluorescent images of each oocyte in each well were taken (at x40 magnification) using the Leica DMBL Upright fluorescence microscope (Leica) and Zen Blue software, capturing with blue and violet light. The images were saved as TIF files.

5.2.11. Assigning cumulus coverage and measuring mean COC diameter

COCs from IVM experiments that had matured at least 120 oocytes (30 per treatment) were assessed for cumulus coverage and expansion (diameter). Images from different dates and wells were randomised prior to this assessment. Therefore, the analyses were carried out blind to avoid biases.

The TIF files of the COCs were opened with ImageJ (64). Each COC was assigned one of three categories; "Full" cumulus coverage, "Partial" cumulus coverage and "No" cumulus coverage (**Figure 5.2**). COCs with Full coverage were completely covered with more than three layers of cumulus cells (**Figure 5.2a**). COCs with partial coverage either did not expand more than three layers or were not completely covered but had at least half of their circumference covered (**Figure 5.2b**). COCs with no coverage had less than half of their circumference covered with cumulus cells (**Figure 5.2c**). A scoring system was then set up whereby COCs with Full coverage were given three points, COCs with Partial coverage were given two points and COCs with No coverage were given one point.

COCs were measured at four points; at 0° (vertical point), 45°, 90° (horizontal point) and 135° (see **Figure 5.3**), using ImageJ (64). Each COC had four diameter measurements in which mean diameter was obtained.

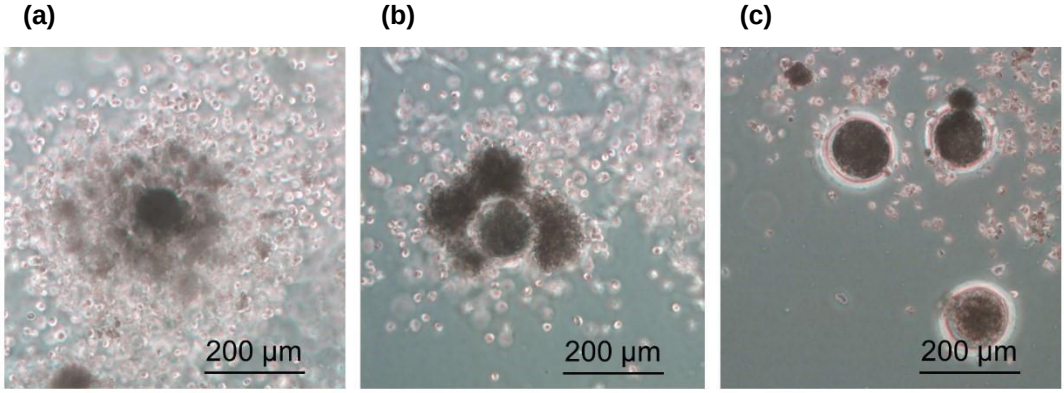


Figure 5.2. Representative images of the patterns of cumulus coverage following 44-hour *in vitro* maturation.

The different categories of cumulus expansion; (a) “Full” cumulus coverage with more than three layers of cumulus cells surrounding the full circumference of the oocytes, (b) “Partial” cumulus coverage where the oocytes have uneven expansion and/or very little expansion with only three or less layers of cumulus cells, (c) “No” cumulus coverage with less than half of their circumference covered with cumulus cells.

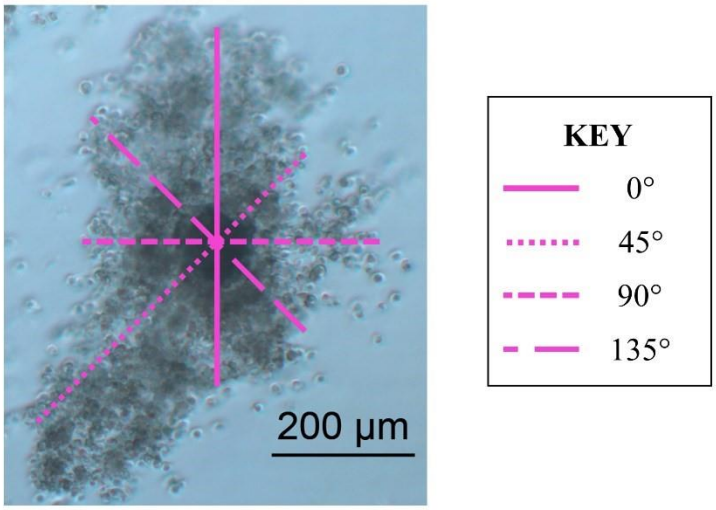


Figure 5.3. Representative image showing the different points on a cumulus-oocyte-complex that were measured to obtain an average diameter.

This is a representative cumulus-oocyte-complex (COC), with the 200 µm scale bar next to it. The pink lines show where the lengths of the COC were measured. The unbroken line is at 0° (vertical position), the small dashed line is at 45°, the medium dashed line is at 90° (horizontal position) and the large dashed line is at 135°. A mean diameter for the COC was obtained from the four diameter measurements.

5.2.12. Assigning stained oocytes with stages of maturation

The method by which the maturation stages of oocytes were assigned was as described by Prentice-Biensch *et al.*, (2012). Briefly, images of the stained oocytes were randomised and each one was assigned one of four stages; germinal vesicle (GV), germinal vesicle breakdown (GVBD), MI and MII. MII oocytes were characterised with two small blue spherical stains that represent the two nuclei (one from the oocyte, the other from the polar body), whilst MI oocytes only had one small spherical stain. The oocytes at GV had extensive nuclear blue staining with green borders surrounding the blue whilst oocytes at GVBD had reduced green staining. From the total number of oocytes that survived the fixing and staining procedure the percentage of oocytes that reached four stages of meiosis (MII, MI, GVBD and GV) were calculated.

5.2.13. Statistical analyses

All statistical tests were carried out on Minitab 17.

Tests for normal distribution of data

All data were subjected to Anderson-Darling normality tests before statistical analyses. Further outlier tests were carried out on data that were not normally distributed. Outliers were removed and tested for normality until a normal distribution was achieved. If non-normal data did not have any outliers or removing outliers did not result in a normal distribution, data were then transformed by one of five functions; $\log^{10}$, squared, cubed, square-root and cube-root. Further Anderson-Darling and Grubb's tests were carried out on transformed data. If after this process the data were not normally distributed, nonparametric tests were used to analyse them. If the transformation removed more than five data points, then this transformation was not used.

Cumulus coverage categories

From the IVM experiments that matured at least 120 oocytes (30 per treatment), the COCs were assigned a category of cumulus coverage and related score; COCs with

Full, Partial or No cumulus coverage were given a score of three, two or one respectively (see section 5.2.11). For each well of each IVM experiment, the mean score was calculated. The resultant values were normally distributed, and therefore General Linear Model analysis of variance (ANOVA) tests were carried out to assess whether there was an effect of linoleic acid treatment. An additional post-hoc analysis was carried out with Tukey Pairwise Comparisons. The General Linear Model ANOVA and Tukey Pairwise Comparisons included the date of the experiment and the well the COC were matured in as well as the treatment as factors in the analyses.

Mean COC diameter

The average diameter of each COC from each IVM experiment was determined as described in section 5.2.11. A mean COC diameter was then obtained for each well. Anderson-Darling normality tests confirmed the normal distribution of the combined data and for each linoleic acid concentration. General linear model ANOVA was then carried out to assess whether there was an overall treatment (linoleic acid) effect on the COC diameter. An additional post-hoc analysis was carried out with Tukey Pairwise Comparisons. The General Linear Model ANOVA and Tukey Pairwise Comparisons included the date of the experiment and the well the COC were matured in as well as the treatment as factors in the analyses.

Oocyte maturation stages

For IVM experiments that retrieved at least 20 oocytes per treatment after fixing and staining, the percentage of oocytes that reached four stages of maturation (MII, MI, GVBD and GV) was calculated for each experiment. Percentages at the GV stage were normally distributed. Squaring the percentages at MII, cube-rooting the percentages at MI and logging (to the power of ten) the percentages at GVBD provided normal distributions. General linear model ANOVA were carried out on combined MII, MI, GVBD and GV percentages as well as between paired treatment groups. An additional post-hoc analysis was carried out with Tukey Pairwise Comparisons. The General Linear Model ANOVA and Tukey Pairwise Comparisons included the date of the

experiment and the well the COC were matured in as well as the treatment as factors in the analyses.

5.3. Results

5.3.1. Cumulus expansion and oocyte maturation was hindered with a high concentration of linoleic acid

In total, 1280 abattoir derived pig COCs were cultured for 44–46 hours in TCM 199 based maturation medium supplemented with different concentrations of linoleic acid to observe the effect of the fatty acid on different parameters of oocyte maturation. In total, 320 COCs were cultured in each treatment. Ten IVM experiments were carried out, each yielding different numbers of COCs and stained oocytes after the protocol (**Table 5.1**). Certain dates (such as the dates in late May) retrieved better quality COCs than earlier dates. Therefore, this was considered in subsequent statistical analyses by fitting the date into ANOVA analyses as described in section 5.2.14.

Maturation in low and medium amounts (50 and 100 µM) of linoleic acid resulted in COCs with similar cumulus expansion and morphology to COCs matured in control medium. However, COCs that were matured in a high concentration of linoleic acid (200 µM) showed reduced cumulus expansion whereby the cells appeared to cluster together and were darker in colour compared to the COCs cultured in lower concentrations of linoleic acid (**Figure 5.4**).

Supplementation of the maturation medium with high linoleic acid concentration was also associated with the decrease in cumulus coverage ($p=0.024$). In particular, the mean±standard error of the mean (SEM) cumulus coverage score was significantly different between COCs matured in control medium (2.60±0.06) compared with COCs matured in medium supplemented with 200 µM linoleic acid (2.30±0.11, **Figure 5.5a**). There was no effect of well ($p=0.784$) but there was an effect of date ($p=0.005$).

An effect of linoleic acid concentration on mean±SEM COC diameter ($p=0.000$, **Figure 5.5b**) was identified with no effect of date and well ($p=0.087$ and $p=0.430$ respectively). Post-hoc analyses between pairs of treatment groups determined that COCs matured in 200 µM linoleic acid (293.61±16.85 µm) had significantly smaller

diameters compared to COCs matured in control medium (388.31±23.38 µm), 50 µM linoleic acid (408.84±16.35 µm) and 100 µM linoleic acid (377.80±16.89 µm).

The total percentage and the mean percentage of oocytes that reached MII of meiosis decreased with 200 µM linoleic acid supplementation (**Figure 5.6**). From all ten IVM experiments, the total percentage of oocytes that reached MII was similar between control (62%), 50 µM linoleic acid (61%) and 100 µM linoleic acid (61%) treatment groups but was considerably lower (44%) with 200 µM linoleic acid (**Figure 5.6a**). Accordingly, the percentage of oocytes that arrested at the metaphase I and GV stages increased with 200 µM linoleic acid (31% and 18% respectively) compared to control (20% and 8%), 50 µM linoleic acid (19% and 10%) and 100 µM linoleic acid (18% and 10%) treatment groups. The percentage of oocytes that arrested at GVBD remained relatively consistent between control (10%), 50 µM (11%), 100 µM (11%) and 200 µM (7%) linoleic acid. Additionally, General Linear Model and Tukey Comparison analyses revealed the significantly lower mean±SEM percentage of metaphase II oocytes with 200 µM linoleic acid supplementation (49.69±7.18, p=0.017) compared to the control treatment (70.81±2.40, **Figure 5.6b**). The percentage of metaphase I oocytes was also increased with linoleic acid supplementation compared, although this trend was not statistically significant (p=0.071).

Date of IVM	Number of aspirated ovaries	Total number of COCs	Number of COC matured	Number of fixed oocytes
18/04/2017	28	277	152	84
24/04/2017	20	174	96	77
25/04/2017	26	138	60	23
02/05/2017	25	114	72	51
09/05/2017	30	177	128	93
15/05/2017	24	271	160	104
16/05/2017	38	387	160	122
22/05/2017	14	293	160	142
23/05/2017	36	254	160	127
30/05/2017	36	294	132	117
Total	**277**	**2379**	**1280**	**940**

Table 5.1. The number of cumulus-oocyte-complexes and oocytes retrieved from each *in vitro* maturation experiment.

The table lists the dates on which the *in vitro* maturation (IVM) experimental repeats were carried out, along with the number of ovaries collected, the total number of cumulus-oocytecomplexes (COCs) found and matured and the total number of stained oocytes fixed for each date. The dates given were the dates on which oocyte aspiration and maturation began. The total number of ovaries only lists those that were healthy and had follicles that could be aspirated. The COCs matured were the sub-population of the total COCs that had full cytoplasm and at least two layers of cumulus cells. An equal number of COCs were matured in each well/linoleic acid treatment in each IVM experiment. The total number of stained oocytes fixed were those that survived the fixing and staining procedure following maturation.

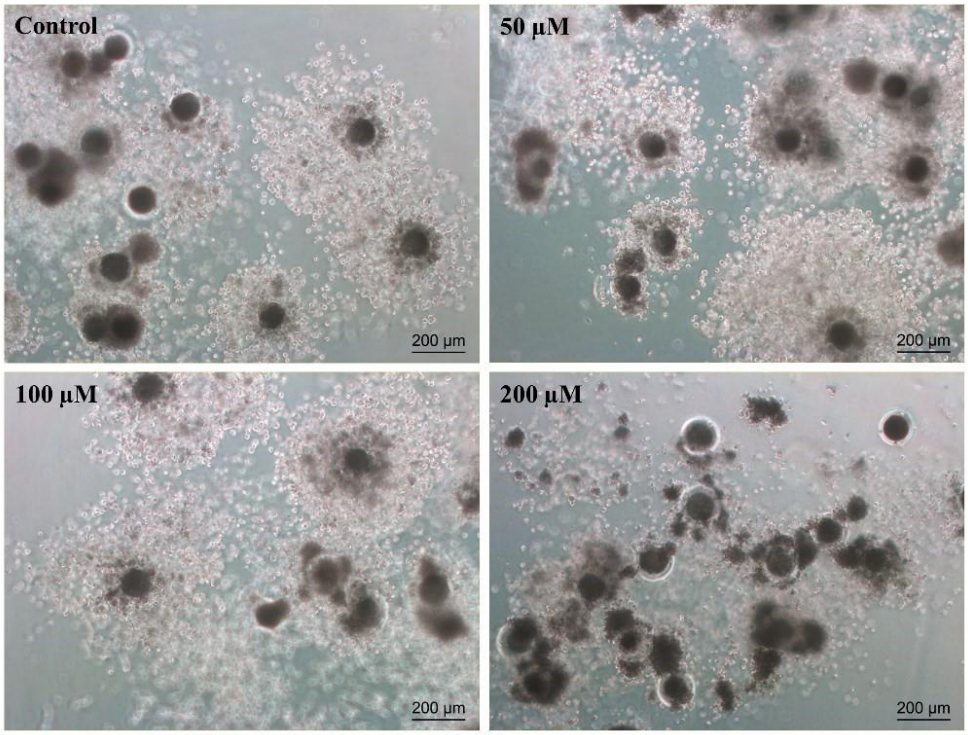


Figure 5.4. Representative images of cumulus-oocyte-complexes matured in control TCM 199 maturation medium and medium supplemented with 0, 50, 100 µM and 200 µM linoleic acid.

Images were taken between 44–46 hours after maturation with Leica DMBL Upright fluorescence microscope and Zen software; scale bars (200 µm) were also applied using Zen Blue software. The cumulus-oocyte-complexes in these images were matured on 18/04/2017 (first day of maturation).

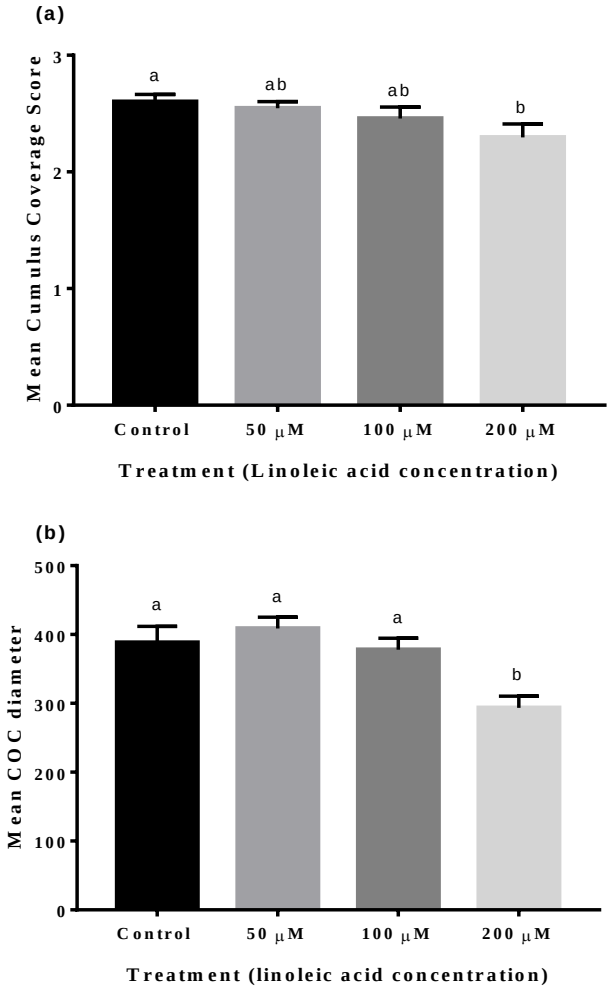


Figure 5.5. Bar charts illustrating the effect of linoleic acid supplementation on cumulus coverage and expansion.

(a) The mean cumulus coverage scores for cumulus-oocyte-complexes (COC) in each linoleic acid treatment; COCs that had Full, Partial and No cumulus coverage were given scores of three, two or one respectively. A mean score was obtained for each well and treatment; no additional linoleic acid (Control) or 50, 100 or 200 μM linoleic acid supplementation. (b) The mean diameter of COCs per well matured for 44 hours in TCM 199 supplemented with different concentrations of linoleic acid. The average diameter for each COC was calculated from four measurements at four different points at 45 ° intervals. Error bars show standard error of the mean. Bars with different letters were significantly different as indicated by General Linear Model ANOVA analysis (and Tukey Pairwise Comparisons) between treatment groups; “a” and “b” denote statistical significance with $p \leq 0.05$.

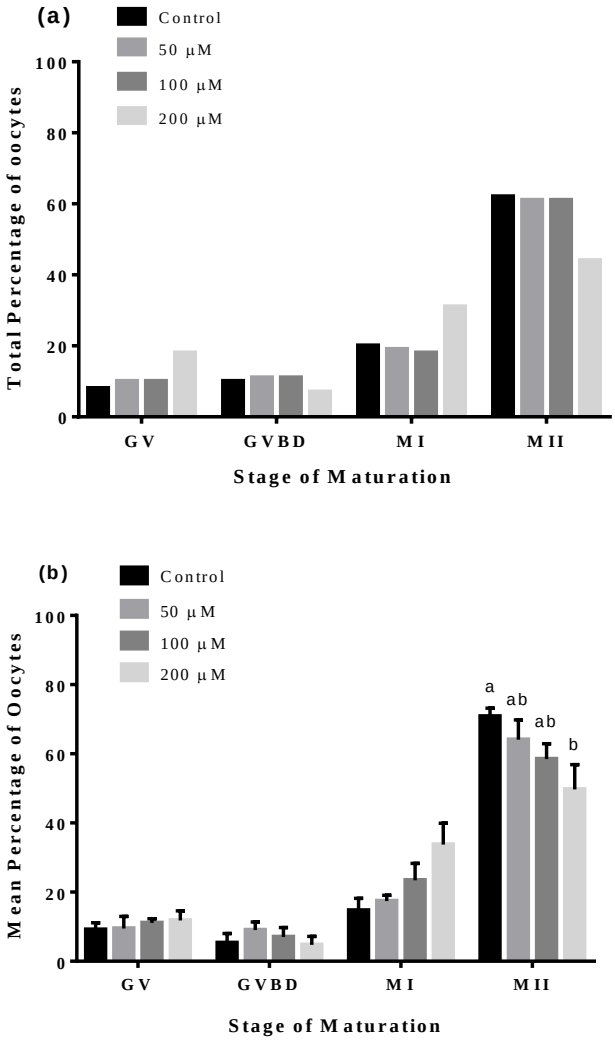


Figure 5.6. Bar charts illustrating the effect of linoleic acid supplementation on oocyte in vitro maturation.

(a) The overall percentage of oocytes from ten experiments and (b) the mean percentage of oocytes per well from five experiments (with at least 20 oocytes per treatment) at different stages of maturation; metaphase II (MII), metaphase I (MI), germinal vesicle breakdown (GVBD) and germinal vesicle (GV) following maturation for 44 hours in TCM 199 supplemented with different concentrations of linoleic acid; no additional linoleic acid (Control) or additional linoleic acid concentration of 50, 100 or 200 µM. Error bars show the standard error of the mean. Bars with different letters were significantly different as indicated by General Linear Model ANOVA analysis (and Tukey Pairwise Comparisons); “a” and “b” denote statistical significance with $p \leq 0.05$.

5.4. Discussion

5.4.1. A high concentration of linoleic acid in maturation medium was detrimental to oocyte maturation and cumulus expansion

In agreement with the bovine studies that observed the detrimental effects of linoleic acid on *in vitro* oocyte maturation, this study observed detrimental effects of a high concentration of linoleic acid on porcine IVM, specifically on cumulus expansion and the proportion of resultant MII oocytes. Unlike the bovine studies that observed detrimental effects at the low concentration of 50 µM, it was only the 200 µM that resulted in the negative effects. However, at this concentration the results mimicked those in bovine studies with the increased proportion of COCs with no expansion, the decreased proportion of COCs that fully expanded, the decreased COC average diameter and the decreased proportion of MII oocytes following IVM (Marei *et al.*, 2010, 2012; Carro *et al.*, 2013; Khalil *et al.*, 2013). Additionally, this was in stark contrast to the effects of *trans*-10, *cis*-12 CLA on porcine COCs, which have been shown to be beneficial at 50 µM but have no effect at 100 µM or 200 µM (Prates *et al.*, 2013a, b; Jia *et al.*, 2014).

The negative effects of linoleic acid were most visible in the cumulus cells. Cumulus cells play a critical role in folliculogenesis and oocyte maturation and are essential for triggering GVBD and re-initiating meiosis (Nevoral *et al.*, 2014). Cumulus cells have been shown to protect bovine oocytes against the potential toxicity of saturated fatty acids by desaturating stearic acid into oleic acid via stearoyl-CoA activity (Aardema *et al.*, 2017). However, linoleic acid is not a saturated fatty acid, so this mechanism was not likely implicated. PFF also contains cumulus expansion enabling factor, although its production of by porcine oocytes mainly occurs during the oocyte's transition to metaphase I (Nagyová *et al.*, 1997; Xingshen *et al.*, 2002). In goat follicles, linoleic acid has been shown to have cumulus-expansion inhibiting activity, but this increased activity involves unknown additional factors (Li *et al.*, 2012b).

Another protective function of cumulus cells is in the protection against reactive oxygen species (ROS), as denuded porcine oocytes exposed to ROS were more likely

to undergo meiotic arrest, degeneration and apoptosis compared to porcine COCs exposed to ROS (Tatemoto *et al.,* 2004). Linoleic acid supplementation of bovine maturation medium also resulted in a disruption in mitochondrial distribution and activity alongside an increase in the production of ROS associated with a decrease in nuclear maturation (Marei *et al.,* 2012). Linoleic acid (along with oleic acid, stearic acid, γ-linolenic acid) has also been shown to stimulate ROS production by human T- and B- lymphocyte-derived cell lines and rat fibroblasts via the activation of nicotinamide adenine dinucleotide phosphate oxidase and protein kinase C (Cury-Boaventura and Curi, 2005; Hatanaka *et al.,* 2013). Therefore, it is likely that the cumulus cells are able to protect the oocyte from the increase in ROS produced from linoleic acid supplementation at 50 µM and 100 µM but that at 200 µM they have exerted a threshold.

5.4.2. The role of linoleic acid in the ovary and in oocyte maturation

Linoleic acid is the precursor for arachidonic acid which is then and converted into prostaglandins through the enzyme action of cycloxegenase 1 and 2 (COX-1 and COX2). Therefore, the relationship between linoleic acid and arachidonic acid has been well documented in relation to reproductive parameters. Women with oocytes that did not show two pronuclei or underwent degeneration have been shown to have had significantly higher FF levels of both arachidonic and linoleic acid derivatives compared to women with normal oocytes (Ciepiela *et al.,* 2015). Intriguingly, the relationship is not always synchronous as arachidonic acid concentration in human FF was shown to be negatively correlated with fertilisation rate whilst linoleic acid was positively correlated (Shaaker *et al.,* 2012).

Diets can also influence intra-ovarian concentrations of linoleic acid which can also affect the downstream molecular pathways. Mice that have been fed a diet rich in linoleic acid had significantly lower arachidonic acid levels in the ovarian phospholipids fractions alongside lower COX-2 protein concentrations and enzyme activity and lower ovarian concentrations of prostaglandin E2 and F2α (Yi *et al.,* 2012). A high-linoleic acid diet significantly increased the ability of both endometrium and placental tissues to produce prostaglandins *in vitro* but as COX protein levels were

unaltered, the main influence was likely to be via conversion of linoleic acid to arachidonic acid, providing an increased supply of precursor (Cheng *et al.*, 2005).

In an IVM system, Jia *et al.*, (2014) found that supplementing oocyte maturation medium with *trans*-10, *cis*-12 CLA increased COX-2 protein levels in COCs which is concurrent with the increase in cumulus expansion and decrease in intracellular ROS in MII oocytes. Therefore, as well as the observable effects of linoleic acid differing with those of *trans*-10, *cis*-12 CLA, perhaps its effects on COX-2 and prostaglandin synthesis would also differ.

5.4.3. Potential avenues for further research

The results of this IVM experiment provided opportunities for further research avenues. As the formation of a blastocyst is the ultimate indicator of a good quality oocyte, IVF experiments could be carried out, as was done in the bovine, ovine and caprine studies (Marei *et al.*, 2010; Carro *et al.*, 2013; Khalil *et al.*, 2013; Amini *et al.*, 2016; Roura *et al.*, 2017). Were it not for the limitations of time and resources, these experiments would have been carried out in addition to the experiments in this study and would have been included in this thesis. Following the results of this study, the hypothesis would be that the blastocyst rate would be significantly lower when the oocytes were matured with 200 µM linoleic acid. It is also possible that there would be a more obvious effect of linoleic acid at 50 µM and 100 µM on blastocyst development. Whilst there were no effects of linoleic acid at these concentrations on cumulus expansion and no statistically significant effect on the resultant MII oocytes following maturation, there was a dose-dependent trend emerging with increasing linoleic acid on the percentage of MII oocytes. These studies would definitively determine whether there were different dosage limits between linoleic acid and its conjugated counterparts.

If time, samples and resources were not restricted, analysis of the media with gas chromatography tandem mass spectrometry may also have been beneficial in measuring the concentration of fatty acids in the media. Gas-chromatography has already been used to measure the concentration of eight fatty acids which were not

measured in this study as well as the oocytes and cumulus cells themselves (Prates *et al.*, 2013a). Measuring the fatty acids in the oocytes and cumulus cells would also give an indication of whether there was an uptake or release of fatty acids specifically from one or both of those cells.

There are also several other components related to cumulus expansion and oocyte maturation that could be measured. Linoleic acid is the precursor to arachidonic acid, which is used to biosynthesise prostaglandins through COX-2 enzyme activity. Prostaglandin E2 and COX-2 also have a direct role in inducing cumulus expansion and oocyte maturation in pigs, cows and mice (Eppig, 1981; Calder *et al.*, 2001; Blaha *et al.*, 2017). Therefore, it would be interesting to measure the concentration of prostaglandins as well as COX-2 enzyme activity in maturation medium following IVM, in a similar way to Jia *et al.*, (2014), following CLA supplementation. Measuring ROS and antioxidants such as glutathione in cumulus cells and medium could also determine whether it is the cumulus cells' role in protecting from these processes that was compromised.

5.4.4. Conclusions

The results of this study provide evidence to suggest the negative impact of a high concentration of linoleic acid on cumulus expansion and oocyte maturation. These results differ greatly from the results of porcine studies assessing the effects of *trans*10, *cis*-12 CLA isomer on porcine IVM. Further work is needed to determine the potential involvement of ROS and COX proteins in this process. This has also opened the opportunity to assess the possibility of supplementing medium with other fatty acids, individually and/or in combination to determine the optimum concentrations of these acids in the oocyte microenvironment. Through this, these conditions could potentially be recreated *in vivo* through the diet.

6. Final Discussion

6.1. Introduction

In this thesis, I hypothesised that a high fibre diet altered the molecular composition of porcine follicular fluid (pFF), which conferred the improvements to oocyte maturation and the subsequent reproductive benefits observed in previous studies. Therefore, the aim of this study was to compare the molecular composition of pFF from high fibrefed pigs with pFF from control-fed pigs, to identify differentially expressed proteins associated with the different diets. Additionally, for both feeding groups, pFF samples were either associated with no blastocyst development or successful blastocyst development following *in vitro* fertilisation (IVF). Therefore, an additional objective was to identify whether these differences were associated with later fertility.

6.2. Experimental Workflow

In this study, a series of experiments were carried out (**Figure 6.1**, purple), beginning with a comparison of the pFF proteome. There were a few alternative directions that could have been taken to achieve the objectives of this study (**Figure 6.1**, pink boxes). For example, measurements or assays for molecules that have already been shown to be altered in plasma as a result of a high fibre diet could have been carried out. These include β-hydroxybutyrate, urea, insulin, dimethyl sulfone, glucose, insulin growth factor-1, creatine and leptin, which were lower in concentration in plasma of high fibre-fed pigs compared to plasma of control-fed pigs whilst levels of short-chain and non-esterified fatty acids were higher (Yde *et al.*, 2011; Weaver *et al.*, 2013; Jégou *et al.*, 2016). However, an omics approach was selected as it had the potential to produce novel hypotheses. The aim was to use proteomics to identify differentially expressed proteins and pathways associated with diet and/or fertility. A subsequent, targeted metabolomic analysis was carried out on a specific sub-set of molecules, influenced by the results of the proteomic analysis.

Differentially expressed proteins were identified between the pooled pFF from pigs fed the different diets and with different IVF outcomes. These proteins were then submitted into Ingenuity Pathway Analysis (IPA) software, which revealed their association with canonical pathways. IPA was selected as it provided several potential research avenues and was heavily used within the institute. However, an alternative pathway software could have also been utilized (**Figure 6.1**), which may have provided further validation to the IPA results.

Candidates were selected that were associated with the detected pathways to (i) confirm the proteomic analyses and (ii) give further indication/confirmation on the potential mechanisms involved in nutrition-mediated reproductive benefits. Due to the limited sample volumes western blots were carried out on pooled samples with the intention to conduct subsequent enzyme-linked immunosorbent assays (ELISAs) to be carried out. The candidates that were confirmed by the western blots were apolipoproteins (A4 and M) associated with cholesterol lipid homeostasis and plasmin/plasminogen involved in proteolytic events at ovulation. However, the ELISA tested for apolipoprotein A4 was not able to detect the protein, implying either a low level of protein for detection or a lack of reactivity/specificity for the pig protein. It could have been possible to forgo the western blots in favour of directly analysing with ELISAs. However, due to interest in sample conservation, it would not have been possible to assay all the candidates in this way.

Due to an interest in the potential involvement of cholesterol/lipid homeostasis, a targeted metabolomic analysis was carried out to measure the concentration of the most abundant fatty acids in pFF. This identified the lower concentration of linoleic acid associated with the high fibre diet. In addition to the metabolomic analysis, further assessment of inflammation could have been carried out, including assays for plasminogen activity or other markers of inflammation. However, due to issues with the apolipoprotein A4 ELISA and the difficulty in finding pig-specific assays, this avenue was not explored. Following the metabolomic analysis, the potential effect of linoleic acid on oocyte *in vitro* maturation (IVM) was assessed. The IVM experiments revealed that supplementation with a high concentration of linoleic acid was

detrimental to cumulus expansion and morphology and oocyte maturation. Alternative IVM experiments (**Figure 6.1**) could have been carried out to test the plasma biomarkers associated with the high fibre diet (Yde *et al.*, 2011; Weaver *et al.*, 2013).

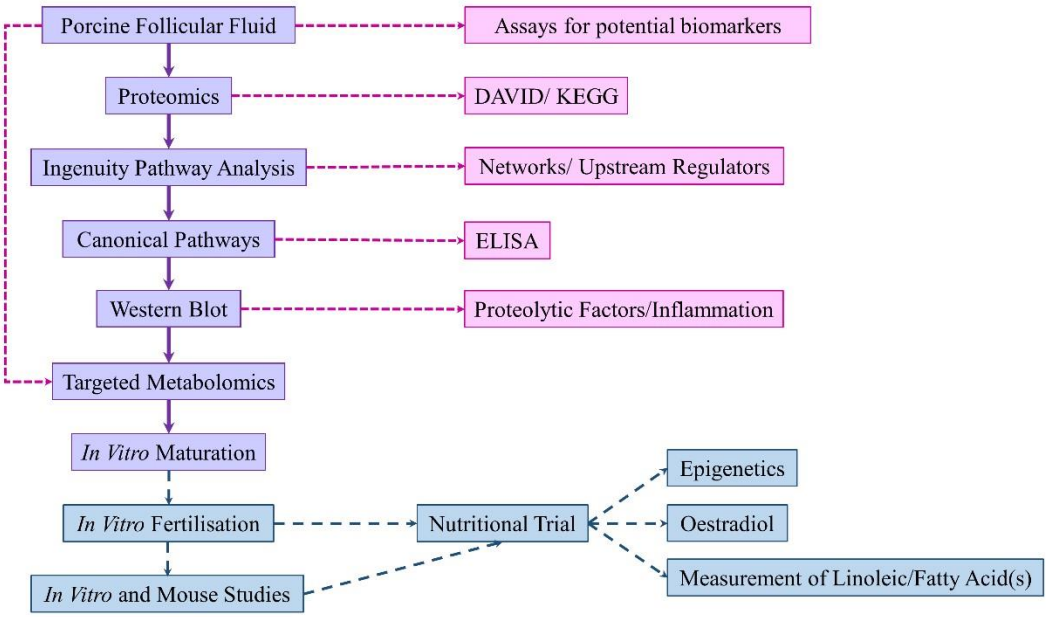


Figure 6.1. Experimental workflow.

Boxes and solid lines in purple show the procedures that were carried out in this study. Pink boxes and dashed lines indicate alternative directions for the study. Blue boxes and dashed lines indicate the potential future studies, following the results of this project and what is known in the literature. Abbreviations: DAVID = Database for Annotation, Visualisation and Integrated Discovery; KEGG = Kyoto Encyclopedia of Genes and Genomes; ELISA = Enzyme-linked immunosorbent assay.

6.3. Potential Mechanisms Involved in Nutritionally Mediated Improvements of Pig Reproduction

Reduction in circulating oestradiol

In addition to insulin and leptin, other hormones affected by the high fibre diet were oestradiol and luteinising hormone (LH), with high fibre-fed gilts having lower circulating oestradiol concentrations on days 17, 18 and 19, increased LH pulse frequency on day 18 and higher LH peaks during their oestrous cycles (Glasgow *et al.*, 1999; Ferguson *et al.*, 2007). The decrease in circulating oestradiol concentrations results in the reduction of the hormone's negative feedback effects on the hypothalamic-pituitary-ovarian (HPG) axis, leading to the delay in the LH surge observed, allowing more time for oocytes to mature prior to ovulation (Ferguson *et al.*, 2007). This delay could have been represented in the reduction of plasmin associated with the high fibre diet, since circulating oestrogen/oestradiol has been correlated with plasmin activation in the mammary secretion of Holstein cows and plasma of postmenopausal women (Athie *et al.*, 1997; Estellés *et al.*, 1999). Additionally, increases in oestradiol levels are negatively correlated with plasminogen inhibitor-1 levels in human plasma and serum and human breast cancer cells, indicating an increase in plasminogen activity associated with increased circulating oestradiol levels (Levenson *et al.*, 1998; Estellés *et al.*, 1999; Magnani *et al.*, 1999).

Oestrogens (including oestradiol) are metabolised in the liver to oestrone and oestriol, and conjugated, making them water soluble. Half of the conjugated oestrogens in the plasma are excreted via the kidney in urine and the other half are excreted into the intestine in bile. Following de-conjugation in the intestinal lumen, about 80% of these oestrogens are reabsorbed through the intestinal wall and vena porta into the liver. However, evidence suggests that the high fibre diet can reduce the reabsorption of oestrogens. In an *in vitro* test system, oestradiol bound strongly to various fibres such as cholestyramin, lignin and cellulose, as well as fibre sources such as wheat bran, cereals, seeds and legumes (Arts *et al.*, 1991b). Additionally, after 24 hours of feeding, the excretion of both free and unconjugated oestrogens in faecal samples was three

times higher in high fibre-fed rats compared to samples from low fibre fed rats (Arts *et al.*, 1991a). The same observation was made with male rats fed high fibre wheat bran, 1 day and 3 weeks after injection with C^{14} labelled oestradiol, compared to male rats fed a non-fibre wheat starch diet or low-fibre wheat flour diet (Arts *et al.*, 1992). Collectively, this indicates that the lower levels of circulating oestradiol could be due to binding of the hormone to the fibres from the sugar beet pulp in the gut, increasing its excretion in faeces and decreasing the levels reabsorbed in the intestines.

The lower circulating levels of oestradiol may also be an indirect result of reduced circulating cholesterol. Cholesterol is converted into progesterone and oestrogens in the ovary during steroidogenesis and dietary fibre is shown to be associated with lower circulating cholesterol levels (van Bennekum *et al.*, 2005; Venkatesan *et al.*, 2007). This can also be corroborated as progesterone concentration was shown to be lower on day 13 of the oestrous cycle in high fibre-fed gilts and their hormone profiles show that they had lower base line progesterone and lower circulating progesterone concentrations (Glasgow *et al.*, 1999; Weaver *et al.*, 2013). However, pFF concentration of oestradiol were unaltered (Ferguson *et al.*, 2007). Therefore, it is less likely that steroidogenesis is the mechanism by which circulating oestradiol concentrations were reduced.

The reverse pattern is also possible whereby lower levels of circulating oestradiol results in the lower levels of circulating cholesterol. Administration of oestradiol to rats increased plasma lipid levels, including high density lipoprotein cholesterol in liver carcinoma cells (Wei *et al.*, 2011). Additionally, selective oestrogen receptor modulators, such as tamoxifen, raloxifen and toremifen, interfered with intracellular cholesterol trafficking and efflux from macrophages of human monocytes (Fernández-Suárez *et al.*, 2016).

The potential effect of lower circulating oestradiol on the apolipoproteins and fatty acids

The lower levels of apolipoproteins associated with high fibre-fed fertile pigs could also indicate lower cholesterol levels. Administration of oestrogen and oestradiol (*in*

vivo and *in vitro*) increased apolipoprotein A4 gene expression in rat brain stems, rat and mouse intestines and rat and mouse livers (Srivastava *et al.*, 1994; Shen *et al.*, 2010, 2014). Additionally, incubation with oestrogen and oestradiol increased mRNA levels of apolipoprotein M in human liver carcinoma cells (Wei *et al.*, 2011, 2017). Therefore, the decrease in circulating oestradiol levels could have led to the decrease in the levels of these apolipoproteins associated high fibre pigs with blastocysts. Oestradiol also affects the levels of other apolipoproteins such as apolipoprotein A1, apolipoprotein B and apolipoprotein E in rat mesenteric lymph, liver and hypocampus (Krause *et al.*, 1981; Seishima *et al.*, 1991; Wang *et al.*, 2006).

In addition to the changes in cholesterol and apolipoproteins, oestradiol supplementation in the form of contraception, increased triglyceride, palmitic acid and oleic acid levels, but also decreased levels of linoleic, arachidonic and eicosapentaenoic acids in women (Sassolas *et al.*, 1983). Additionally, administration of oestradiol to castrated male rats resulted in a marked decrease of linoleic acid in liver microsomal phospholipids (Moore *et al.*, 1977). Therefore, these results are in contradiction to the results of this study as the high fibre diet was associated with the reduction of both circulating oestradiol and pFF levels of linoleic acid.

However, the lower levels of linoleic acid may be what confers the decrease in oestradiol as plasma concentrations of oestradiol were significantly higher in pregnant female rats fed a diet rich in linoleic acid (Hilakivi-Clarke *et al.*, 1997). Additionally, the rise in maternal oestradiol concentrations following induced labour occurred earlier in ewes fed a diet supplemented with linoleic acid compared with ewes fed a control diet (Elmes *et al.*, 2005). Therefore, the differences in levels of linoleic acid may still have been directly due to the diet, but along with the binding of oestrogens in the gut, the lower levels of linoleic acid may also contribute to the lower level of oestradiol. However, since the concentration of oestradiol in pFF does not change (only circulating levels) and there are no measurements for circulating linoleic acid levels, it is difficult to determine whether the lower levels of these molecules, due to the high fibre diet, are directly related.

Alternative epigenetic mechanism involving betaine induced methylation

Oocyte maturation is a crucial period for the deletion, acquisition and conservation of genomic imprints, which are partly mediated by the pattern of gene methylation. Therefore, the metabolic state of females during ovarian follicular growth can exert important effects on the methylation process (Sinclair *et al.*, 2016). Betaine is a derivative of trimethyl glycine, involved in deoxyribonucleic acid (DNA) and histone methylation by donating a methyl group, through a one-carbon process (Anderson *et al.*, 2012). Dietary betaine donates a methyl group to homocysteine, converting it into methionine, and betaine itself is converted into dimethylglycine in a reaction catalysed by the enzyme betaine-homocysteine methyltransferase (BHMT). Methionine is then converted into the methyl donor S-adenosylmethionine, the major methyl donor of DNA methylation (Chiang *et al.*, 1996). In the ovary, betaine is transported through cumulus cells during meiotic maturation, and the betaine in metaphase II oocytes is then regulated by the imino acid transporter that arises post-fertilisation (Corbett *et al.*, 2014). BHMT activity works in conjunction with the folate cycle in methylation processes in oocytes and blastocysts, and the function of these pathways affect the mean number of cells in the inner cell mass of mouse blastocysts (Zhang *et al.*, 2016).

Increased dietary fibre in pigs and humans has been shown to increase plasma betaine concentrations, as well as other molecules in the methylation process such as homocysteine, dimethylglycine and methionine (Bertram *et al.*, 2009; Price *et al.*, 2010; Ross *et al.*, 2011; Hedemann *et al.*, 2015). Plasma betaine levels also increased in mice whose drinking water was supplemented with betaine and increased more when also supplemented with soluble fibre (Pekkinen *et al.*, 2013). There was a study that observed elevated plasma betaine in low fibre-fed sows compared to control and high fibre-fed sows, but this study only included six sows and the diets were fed in a repeated 3 x 3 cross-over design, which could have led to a merging of dietary effects (Yde *et al.*, 2012). Furthermore, the study was carried out by the same researchers that identified betaine as a plasma biomarker for feeding sows sugar beet pulp in a study that incorporated a larger number of animals (12 per treatment group) and without the cross-over design (Yde *et al.*, 2011). Therefore, the increase in plasma betaine could

be represented in the pFF, and potentially be involved in improved oocyte maturation through beneficial DNA methylation.

6.4. Limitations of the Study and Potential Future Research Opportunities

The biggest limitation of this study was the restriction on sample types available. Potential upstream or downstream effects could not be assessed without associated plasma samples, oocytes or surrounding somatic cells. Similar experiments could have been carried out on plasma samples to assess whether there were any positive or negative correlations between protein composition, protein concentrations or fatty acid concentration with their associated pFF. Additionally, compromises were made to the experimental workflow to ensure the conservation of the pFF, available at limited volumes. If there were no sample limitations, individual samples could have been analysed instead of analysing pooled samples in the proteomic analyses and western blots. However, there is little that could have been done to overcome the limited amount of pFF samples. The pFF samples were collected from the 16 largest follicles (presumed ovulatory population), of which equal volumes were taken from each follicle (the volume being the smallest follicle) to make the pFF sample pool for that animal. Fluid could have been collected from all the follicles and pooled to include the total volume from each follicle. However, this would have potentially introduced compositional differences due to follicle size and over-represented larger follicles.

The proteomic study yielded such a large dataset that could be analysed and interpreted in a variety of ways, potentially leading to more comprehensive bioinformatic studies, either using IPA or another bioinformatic approach. In addition to an expansion of the proteomic analyses, further untargeted metabolomic analyses could be carried out to assess the composition of other metabolites, including other forms of lipids. Taken together, the results of this study and in the literature provide several avenues for further research (**Figure 6.1**, blue boxes). The observations from the IVM experiments warrant further investigation of the effects of increased linoleic acid concentration on embryo development through IVF experiments, the results of which could be followed with a nutritional trial. This would provide information on whether the detrimental

effects of a high linoleic acid concentration during maturation extend to subsequent fertilisation rates and/or embryo development.

A mechanism that was proposed was the delay in ovulation as a result of lower circulating oestradiol. *In vitro* systems and rodent studies could be used to measure the potential of different fibre sources to bind to labelled oestradiol and measure cycling oestrogen respectively. The degree of steroid binding *in vitro* has been shown to be inversely proportional to their apparent digestibility in the pig (Arts *et al.*, 1991b). Therefore, the results of these *in vitro* and mouse studies can be followed by an actual nutritional study using both sows and gilts. From an agricultural perspective, byproducts from the food and drink industry such as wheat millrun and oat husks could be compared with sugar beet pulp to identify the most efficient fibre source for binding to oestrogen and lowering its circulating levels. Additionally, litter size, within-litter variation in birth weight, neonatal vigour and survival to weaning could also be compared.

A nutritional trial would provide the opportunity to do many more experiments by collecting other samples such as plasma, oocytes and somatic cells, which were not available for this study. Additionally, quantitative polymerase chain reaction (qPCR) experiments could have been carried out to measure the level of transcription of liver X receptor (LXR), farnesoid X receptor (FXR) and retinoid X receptor (RXR) target genes, thereby measuring their transcriptional activities. Oocytes from IVM experiments and blastocysts from IVF experiments, following the nutritional studies could be used to measure the level of methylation involved. Measurements of betaine in pFF can give an indication of the level of epigenetic alterations that resulted from the high fibre diet.

Once an optimum fibre source is identified, the duration of feeding could also be assessed. The current optimum period for feeding the diet is 19 days prior to predicted ovulation. However, the most effective time period in which the dietary intervention elicits the reproductive benefits is likely to be shorter. Therefore, similar nutritional trials could be carried out whereby the diet is given during different periods of the 19

days prior to ovulation. It may be that the most effective period to feed the diet is during the second half of the oestrous cycle, while the volume of pFF increases.

6.5. Conclusion

Using a proteomic approach, protein candidates were identified as being differentially expressed between pooled pFF samples from pigs fed different diets (control versus high fibre) and associated with different IVF outcomes (with or without a blastocyst). The protein candidates were associated with canonical pathways involved in inflammation (acute phase response and coagulation) and cholesterol/lipid homeostasis (LXR/RXR and FXR/RXR activation). These protein candidates included plasminogen, and its active form plasmin, a proteolytic enzyme involved in weakening the follicular wall prior to ovulation. The lower expression of plasminogen and plasmin in pFF of high fibre pigs implies a delay in the accumulation of the inflammatory proteins required for ovulation. The other protein candidates were apolipoprotein A4 and apolipoprotein M, whereby the increase in apolipoproteins associated with blastocyst development was only observed with pFF of control pigs but not high fibre pigs. Targeted metabolomic analyses identified the lower concentration of linoleic acid in pFF of high fibre-fed pigs, which could potentially be beneficial to oocyte maturation, as shown by the negative effects of a high linoleic acid concentration on oocyte IVM.

Previous studies showed the decrease in circulating concentration of oestradiol prior to ovulation in pigs fed the high fibre diet, most likely due to increased binding of oestrogens to fibre in the gut. The decrease in oestradiol can be represented by the lack of accumulation of apolipoprotein A4 and apolipoprotein M, as mRNA expression of these proteins can be enhanced by oestradiol. The decrease in circulating oestradiol interrupts the negative feedback on the HPG axis, thereby delaying the LH surge and ovulation and prolonging oocyte maturation, leading to more mature oocytes. The lengthening of oocyte maturation could be represented by the delay in accumulation of the proteolytic enzymes, such as plasmin, in preparation of ovulation.

Further studies are required to elucidate the intricacies of this proposed mechanism (**Figure 6.2**) and to identify whether unmolassed sugar beet pulp is the most efficient at stimulating this mechanism. Additionally, the potential of alternative mechanisms involved in improved oocyte maturation could be assessed, such as the involvement of betaine induced DNA methylation.

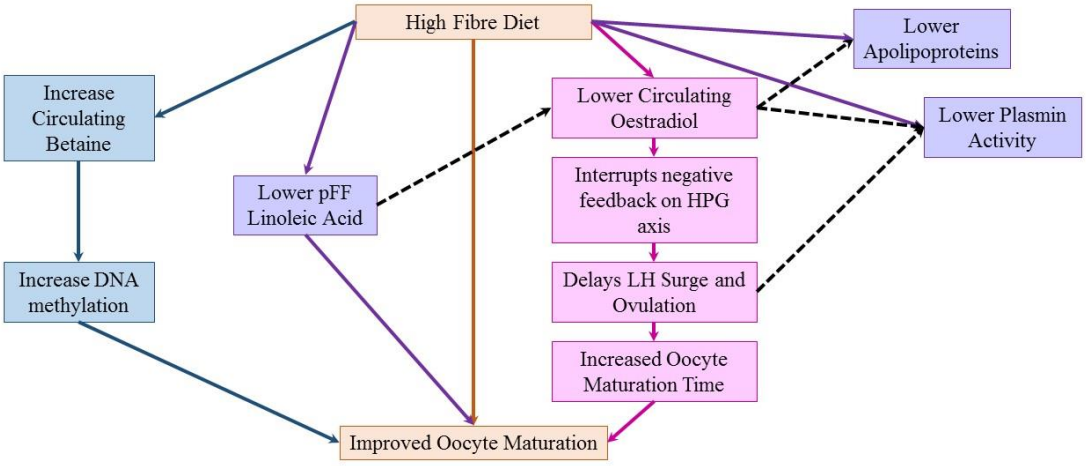


Figure 6.2. Potential mechanism.

Boxes in purple are summarised results carried out in this study. Pink boxes are results and propositions made by Ashworth *et al.*, (2006) and Ferguson *et al.*, (2007b). Blue boxes propose an alternative mechanism involving betaine induced methylation. Grey dash lines indicate proposed associations between the results of this study and the results of previous studies. Abbreviations: DNA = deoxyribonucleaic acid; pFF = porcine follicular fluid; HPG = hypothalamic-pituitary-gonadal, LH = luteinising hormone.

References

Aardema H, Lolicato F, van de Lest CHA, Brouwers JF, Vaandrager AB, van Tol HTA, Roelen BAJ, Vos PLAM, Helms JB and Gadella BM (2013) Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. *Biology of Reproduction* **88** 1–15.

Aardema H, Gadella BM, van de Lest CH, Brouwers JF, Stout TA, Roelen BA and Vos PL (2015) Free fatty acid levels in fluid of dominant follicles at the preferred insemination time in dairy cows are not affected by early postpartum fatty acid stress. *Journal of Dairy Science* **98** 2322–2336.

Aardema H, van Tol HTA, Wubbolts RW, Brouwers JFHM, Gadella BM and Roelen BAJ (2017) Stearoyl-CoA desaturase activity in bovine cumulus cells protects the oocyte against saturated fatty acid stress. *Biology of Reproduction* **96** 982–992.

Absalón-Medina VA, Bedford-Guaus SJ, Gilbert RO, Siqueira LC, Esposito G, Schneider A, Cheong SH and Butler WR (2014) The effects of conjugated linoleic acid isomers cis- 9,trans- 11 and trans- 10,cis- 12 on in vitro bovine embryo production and cryopreservation. *Journal of Dairy Science* **97** 6164–6176.

Ahnström J, Lindqvist PG, Walle U and Dahlbäck B (2010) Plasma levels of apolipoprotein M in normal and complicated pregnancy. *Acta Obstetricia et Gynecologica* **89** 1214–1217.

Ainsworth L, Baker RD and Armstrong DT (1975) Pre-ovulatory changes in follicular fluid prostaglandin F levels in swine. *Prostaglandins* **9** 915–925.

Ainsworth L, Tsang BK, Downey BR, Marcus GJ and Armstrong DT (1980) Interrelationships between follicular fluid steroid levels, gonadotropic stimuli, and oocyte maturation during preovulatory development of porcine follicles. *Biology of Reproduction* **23** 621–627.

Altomare A, Fasoli E, Colzani M, Parra PMX, Ferrari M, Cilurzo F, Rumio C, Cannizzaro L, Carini M, Giorgio P *et al.* (2016) An in depth proteomic analysis based on ProteoMiner, affinity chromatography and nano-HPLC-MS/MS to explain the potential health benefits of bovine colostrum. *Journal of Pharmaceutical and Biomedical Analysis* **121** 297–306.

Alviggi C, Humaidan P, Howles CM, Tredway D and Hillier SG (2009) Biological versus chronological ovarian age: implications for assisted reproductive technology. *Reproductive Biology and Endocrinology* **7** 1–13.

Ambekar AS, Nirujogi RS, Srikanth SM, Chavan S, Kelkar DS, Hinduja I, Zaveri K, Prasad TSK, Harsha HC, Pandey A *et al.* (2013) Proteomic analysis of human follicular fluid: A new perspective towards understanding

folliculogenesis. *Journal of Proteomics* **87** 68–77.

Ambekar AS, Kelkar DS, Pinto SM, Sharma R, Hinduja I, Zaveri K, Pandey A, Prasad TSK, Gowda H and Mukherjee S (2015) Proteomics of follicular fluid from women with polycystic ovary syndrome suggests molecular defects in follicular development. *Journal of Clinical Endocrinology and Metabolism* **100** 744–753.

Amini E, Asadpour R, Roshangar L and Jafari-Joozani R (2016) Effect of linoleic acid supplementation on in vitro maturation, embryo development and apoptotic related gene expression in ovine. *International Journal of Reproductive Biomedicine* **14** 255–262.

Ampatzoglou A, Williams CL, Atwal KK, Maidens CM, Ross AB, Thielecke F, Jonnalagadda SS, Kennedy OB and Yaqoob P (2016) Effects of increased wholegrain consumption on immune and inflammatory markers in healthy low habitual wholegrain consumers. *European Journal of Nutrition* **55** 183–195.

Anahory T, Dechaud HH, Bennes RR, Martin P, Lamb NJ and Laoudj D (2002) Identification of new proteins in follicular fluid of mature human follicles. *Electrophoresis* **23** 1197–1202.

Anaya-Hernández A, Méndez-Tepepa M, Hernández Aragón LG, Pacheco P, Martínez-Gómez M, Castelán F and Cuevas E (2014) Farnesoid X receptor immunolocalization in reproductive tissues of adult female rabbits. *Acta Histochemica* **116** 1068–1074.

Anderson OS, Sant KE and Dolinoy DC (2012) Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *Journal of Nutritional Biochemistry* **23** 853–859.

Angelucci S, Ciavardelli D, Di Giuseppe F, Eleuterio E, Sulpizio M, Tiboni GM, Giampietro F, Palumbo P and Di Ilio C (2006) Proteome analysis of human follicular fluid. *Biochimica et Biophysica Acta* **1764** 1775–1785.

Arias-Álvarez M, García-García R, Rebollar P, Nicodemus N, Millán P, Revuelta L and Lorenzo P (2010) Follicular, oocyte and embryo features related to metabolic status in primiparous lactating does fed with high-fibre rearing diets. *Reproduction in Domestic Animals* **45** e91–e100.

Arts CJM, de Bie ATJJ, van den Berg H, van't Veer P, Bunnik GSJ and Thijssen JHH (1991a) Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats. *Journal of Steroid Biochemistry and Molecular Biology* **39** 193–202.

Arts CJM, Govers CARL, van den Berg H, Wolters MGE, van Leeuwen P and Thijssen JHH (1991b) In vitro binding of estrogens by dietary fiber and the in vivo apparent digestibility tested in pigs. *Journal of Steroid Biochemistry and Molecular Biology* **38** 621–628.

Arts CJM, Govers CARL, van den Berg H, Blankenstein MA and Thijssen JHH (1992) Effect of wheat bran on excretion of radioactively labeled estradiol-17β estrone-glucoronide injected intravenously in male rats. *Journal of Steroid Biochemistry and Molecular Biology* **42** 103–111.

Ashworth CJ, Ferguson EM, Edwards SA and Hunter MG (2008) Nutritional insights into the origins of embryonic loss in the pig. In *Proceedings of the Second Workshop on Embryonic and Fetal Nutrition, Ravello, Italy, 29 May-1 June 2006*, pp 27–29. Eds S Wilsher, W Allen and J Wade. Newmarket, UK: R & W Communications.

Ashworth CJ, Toma LM and Hunter MG (2009) Nutritional effects on oocyte and embryo development in mammals: implications for reproductive efficiency and environmental sustainability. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **364** 3351–3361.

Athie F, Bachman KC, Head HH, Hayen MJ and Wilcox CJ (1997) Milk plasmin during bovine mammary involution that has been accelerated by estrogen. *Journal of Dairy Science* **80** 1561–1568.

Bagg MA, Nottle MB, Armstrong DT and Grupen CG (2007) Relationship between follicle size and oocyte developmental competence in prepubertal and adult pigs. *Reproduction, Fertility and Development* **19** 797–803.

Banni S (2002) Conjugated linoleic acid metabolism. *Current Opinion in Lipidology* **13** 261–266.

Basini G, Simona B, Santini SE and Grasselli F (2008) Reactive oxygen species and anti-oxidant defences in swine follicular fluids. *Reproduction, Fertility and Development* **20** 269–274.

Beers WH (1975) Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. *Cell* **6** 379–386.

Bender K, Walsh S, Evans ACO, Fair T and Brennan L (2010) Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. *Reproduction* **139** 1047–1055.

van Bennekum AM, Nguyen D V, Schulthess G, Hauser H and Phillips MC (2005) Mechanisms of cholesterol-lowering effects of dietary insoluble fibres: relationships with intestinal and hepatic cholesterol parameters. *The British Journal of Nutrition* **94** 331–337.

Berendt FJ, Fröhlich T, Bolbrinker P, Boelhauve M, Güngör T, Habermann FA, Eckhard W and Arnlod GJ (2009) Highly sensitive saturation labeling reveals changes in abundance of cell cycle-associated proteins and redox enzyme variants during oocyte maturation in vitro. *Proteomics* **9** 550–564.

Bertoldo MJ, Nadal-Desbarats L, Gérard N, Dubois A, Holyoake PK and Grupen CG (2013) Differences in the metabolomic signatures of porcine

follicular fluid collected from environments associated with good and poor oocyte quality. *Reproduction* **146** 221–231.

Bertram HC, Malmendal A, Nielsen NC, Straadt IK, Larsen T, Knudsen KEB and Lærke HN (2009) NMR-based metabonomics reveals that plasma betaine increases upon intake of high-fiber rye buns in hypercholesterolemic pigs. *Molecular Nutrition and Food Research* **53** 1055–1062.

Bianchi L, Gagliardi A, Campanella G, Landi C, Capaldo A, Carleo A, Armini A, De Leo V, Piomboni P, Focarelli R *et al.* (2013) A methodological and functional proteomic approach of human follicular fluid en route for oocyte quality evaluation. *Journal of Proteomics* **90** 61–76.

Biggs C, Tilton JE, Craigon J, Foxcroft GR, Ashworth CJ and Hunter MG (1993) Comparison of follicular heterogeneity and ovarian characteristics in Meishan and Large-White hybrid pigs. *Journal of Reproduction and Fertility* **97** 263–269.

Bijttebier J, Tilleman K, Dhaenens M, Deforce D, Van Soom A and Maes D (2009) Comparative proteome analysis of porcine follicular fluid and serum reveals that excessive alpha(2)-macroglobulin in serum hampers successful expansion of cumulus-oocyte complexes. *Proteomics* **9** 4554–4565.

Bindelle J, Leterme P and Buldgen A (2008) Nutritional and environmental consequences of dietary fibre in pig nutrition: a review. *Biotechnologie, Agronomie, Société et Environnement* **12** 69–80.

Black JL and Erickson BH (1968) Oogenesis and ovarian development in the prenatal pig. *The Anatomical Record* **161** 45–55.

Blaha M, Prochazka R, Adamkova K, Nevoral J and Nemcova L (2017) Prostaglandin E2 stimulates the expression of cumulus expansion-related genes in pigs: the role of protein kinase B. *Prostaglandins and Other Lipid Mediators* **130** 38–46.

Bokal EV, Tacer KF, Vrbnjak M, Lepoša S, Virant-Klun I, Verdenik I and Rozman D (2006) Follicular sterol composition in gonadotrophin stimulated women with polycystic ovarian syndrome. *Molecular and Cellular Endocrinology* **249** 92–98.

Boots CE and Jungheim ES (2015) Inflammation and human ovarian follicular dynamics. *Seminars in Reproductive Medicine* **33** 270–275.

Brankin V, Hunter MG, Horan TL, Armstrong DG and Webb R (2004) The expression patterns of RNA-encoding stem cell factor, internal stem cell factor and c-kit in the prepubertal and adult porcine ovary. *Journal of Anatomy* **205** 393–403.

Brankin V, Quinn RL, Webb R and Hunter MG (2005) Evidence for a functional bone morphogenetic protein (BMP) system in the porcine ovary. *Domestic*

Animal Endocrinology **28** 367–379.

Broderick GA, Faciola AP and Armentano LE (2015) Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows. *Journal of Dairy Science* **98** 5672–5687.

Budna J, Celichowski P, Karimi P, Kranc W, Bryja A, Ciesiółka S, Rybska M, Borys S, Jeseta M, Bukowska D *et al.* (2017a) Does porcine oocytes maturation in vitro is regulated by genes involved in transforming growth factor beta receptor signaling pathway? *Advances in Cell Biology* **5** 1–14.

Budna J, Chachuta A, Kaźmierczak D, Rybska M, Ciesiółka S, Bryja A, Kranc W, Borys S, Żok A, Bukowska D *et al.* (2017b) Morphogenesis-related gene-expression profile in porcine oocytes before and after in vitro maturation. *Zygote* **25** 331–340.

Büscher U, Chen FCK, Kentenich H and Schmiady H (1999) Cytokines in the follicular fluid of stimulated and non-stimulated human ovaries; is ovulation a suppressed inflammatory reaction? *Human Reproduction* **14** 162–166.

Calder MD, Caveney AN, Westhusin ME and Watson AJ (2001) Cyclooxygenase-2 and prostaglandin E2(PGE2) receptor messenger RNAs are affected by bovine oocyte maturation time and cumulus-oocyte complex quality, and PGE2 induces moderate expansion of the bovine cumulus in vitro. *Biology of Reproduction* **65** 135–140.

Cárdenas H, Herrick JR and Pope WF (2002) Increased ovulation rate in gilts treated with dihydrotestosterone. *Reproduction* **123** 527–533.

Carro M, Buschiazzo J, Ríos GL, Oresti GM and Alberio RH (2013) Linoleic acid stimulates neutral lipid accumulation in lipid droplets of maturing bovine oocytes. *Theriogenology* **79** 687–694.

Castañeda-Gutiérrez E, Benefield BC, de Veth MJ, Santos NR, Gilbert RO, Butler WR and Bauman DE (2007) Evaluation of the mechanism of action of conjugated linoleic acid isomers on reproduction in dairy cows. *Journal of Dairy Science* **90** 4253–4264.

Castells L, Bach A and Terré M (2015) Short- and long-term effects of forage supplementation of calves during the preweaning period on performance, reproduction, and milk yield at first lactation. *Journal of Dairy Science* **98** 4748–4753.

Cesarman-Maus G and Hajjar KA (2005) Molecular mechanisms of fibrinolysis. *British Journal of Haematology* **129** 307–321.

Chakraborty S and Shekhar HU (2017) Applications of mass-spectrometry based quantitative proteomics to understand complex cellular functions and cell fate decisions. *Biomedical Journal of Scientific and Technical Research* **1** 1–3.

Chang CS, Jones D, Ellefson RD and Ryan RJ (1976) The porcine ovarian follicle: I. Selected chemical analysis of follicular fluid at different developmental stages. *Biology of Reproduction* **15** 321–328.

Che L, Feng D, Wu D, Fang Z, Lin Y and Yan T (2011) Effect of dietary fibre on reproductive performance of sows during the first two parities. *Reproduction in Domestic Animals* **46** 1061–1066.

Cheng Z, Elmes M, Kirkup SE, Chin EC, Abayasekara DRE and Wathes DC (2005) The effect of a diet supplemented with the n-6 polyunsaturated fatty acid linoleic acid on prostaglandin production in early- and late-pregnant ewes. *Journal of Endocrinology* **184** 165–178.

Chiang PK, Gordon K, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K and McCann PP (1996) S-Adenosylmethionine and methylation. *The FASEB Journal* **10** 471–480.

Childs S, Hennessy AA, Sreenan JM, Wathes DC, Cheng Z, Stanton C, Diskin MG and Kenny DA (2008) Effect of level of dietary n-3 polyunsaturated fatty acid supplementation on systemic and tissue fatty acid concentrations and on selected reproductive variables in cattle. *Theriogenology* **70** 595–611.

Ciepiela P, Bączkowski T, Drozd A, Kazienko A, Stachowska E and Kurzawa R (2015) Arachidonic and linoleic acid derivatives impact oocyte ICSI fertilization – A prospective analysis of follicular fluid and a matched oocyte in a 'one follicle – one retrieved oocyte – one resulting embryo' investigational setting. *PLOS ONE* **10** 1–14.

Clarke HG, Hope SA, Byers S and Rodgers RJ (2006) Formation of ovarian follicular fluid may be due to the osmotic potential of large glycosaminoglycans and proteoglycans. *Reproduction* **132** 119–131.

Clayton L and Johnson MH (1998) Tropomyosin in preimplantation mouse development: identification, expression, and organization during cell division and polarization. *Experimental Cell Research* **238** 450–464.

Cook B, Hunter RH and Kelly AS (1977) Steroid-binding proteins in follicular fluid and peripheral plasma from pigs, cows and sheep. *Journal of Reproduction & Fertility* **51** 65–71.

Corbett HE, Dubé CD, Slow S, Lever M, Trasler JM and Baltz JM (2014) Uptake of betaine into mouse cumulus-oocyte complexes via the SLC7A6 isoform of y+L transporter. *Biology of Reproduction* **90** 1–9.

Cordeiro FB, Cataldi TR, Perkel KJ, da Costa L do VT, Rochetti RC, Stevanato J, Eberlin MN, Zylbersztejn DS, Cedenho AP and Lo Turco EG (2015) Lipidomics analysis of follicular fluid by ESI-MS reveals potential biomarkers for ovarian endometriosis. *Journal of Assisted Reproduction and Genetics* **32** 1817–1825.

Cury-Boaventura MF and Curi R (2005) Regulation of reactive oxygen species (ROS) production by C18 fatty acids in Jurkat and Raji cells. *Clinical Science* **108** 245–253.

Dai G and Lu G (2012) Different protein expression patterns associated with polycystic ovary syndrome in human follicular fluid during controlled ovarian hyperstimulation. *Reproduction, Fertility, and Development* **24** 893–904.

Dang-Nguyen TQ, Haraguchi S, Kikuchi K, Somfai T, Bodó S and Nagai T (2014) Leukemia inhibitory factor promotes porcine oocyte maturation and is accompanied by activation of signal transducer and activator of transcription 3. *Molecular Reproduction and Development* **81** 230–239.

Darroch CS, Dove CR, Maxwell C V, Johnson ZB and Southern LL (2008) A regional evaluation of the effect of fiber type in gestation diets on sow reproductive performance. *Journal of Animal Science* **86** 1573–1578.

DeDecker AE, Hanson AR, Walker PM and Salak-Johnson JL (2014) Space allowance and high fiber diet impact performance and behavior of group-kept gestating sows. *Journal of Animal Science* **92** 1666–1674.

Delgado R, Abad-Guamán R, Nicodemus N, Villamide MJ, Ruiz-López N, Carabaño R, Menoyo D and García J (2018) Effect of level of soluble fiber and n-6/n-3 fatty acid ratio on performance of rabbit does and their litters. *Journal of Animal Science* **96** 1084–1100.

Dieplinger H, Ankerst DP, Burges A, Lenhard M, Lingenhel A, Fineder L, Buchner H and Stieber P (2009) Afamin and apolipoprotein A-IV: Novel protein markers for ovarian cancer. *Cancer Epidemiology, Biomarkers and Prevention* **18** 1127–1134.

Ding J and Foxcroft GR (1994) Epidermal growth factor enhances oocyte maturation in pigs. *Molecular Reproduction and Development* **39** 30–40.

Ding P-H and Jin L (2014) The role of lipopolysaccharide-binding protein in innate immunity: a revisit and its relevance to oral/periodontal health. *Journal of Periodontal Research* **49** 1–9.

Do LTK, Shibata Y, Taniguchi M, Nii M, Nguyen T V, Tanihara F, Takagi M and Otoi T (2015) Melatonin supplementation during in vitro maturation and development supports the development of porcine embryos. *Reproduction in Domestic Animals* **50** 1054–1058.

Dorshorst ME and Grummer RR (2002) Effects of day relative to parturition and dietary crude protein on rumen fermentation in prepartum transition cows. *Journal of Dairy Science* **85** 2290–2298.

Drouineaud V, Sagot P, Garrido C, Logette E, Deckert V, Gambert P, Jimenez C, Staels B, Lagrost L and Masson D (2007) Inhibition of progesterone production in human luteinized granulosa cells treated with LXR agonists.

Molecular Human Reproduction **13** 373–379.

Ducolomb Y, González-Márquez H, Fierro R, Jiménez I, Casas E, Flores D, Bonilla E, Salazar Z and Betancourt M (2013) Effect of porcine follicular fluid proteins and peptides on oocyte maturation and their subsequent effect on in vitro fertilization. *Theriogenology* **79** 896–904.

Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL and Schoolcraft WB (2014) Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health. *Fertility and Sterility* **103** 303–316.

Dunlop CE and Anderson RA (2014) The regulation and assessment of follicular growth. *Scandinavian Journal of Clinical and Laboratory Investigation. Supplementum* **74** 13–17.

Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ and Robker RL (2010) Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biology of Reproduction* **83** 909–918.

Dunning KR, Russell DL and Robker RL (2014) Lipids and oocyte developmental competence: the role of fatty acids and β-oxidation. *Reproduction* **148** R15–R27.

Dwivedi RC, Krokhin O V., Cortens JP and Wilkins JA (2010) Assessment of the reproducibility of random hexapeptide peptide library-based protein normalization. *Journal of Proteome Research* **9** 1144–1149.

Eaton SL, Roche SL, Hurtado ML, Oldknow KJ, Farquharson C, Gillingwater TH and Wishart TM (2013) Total protein analysis as a reliable loading control for quantitative fluorescent western blotting. *PLOS ONE* **8** e72457.

Edwards RG (1965) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* **208** 349–351.

Edwards LJ, Williams DA and Gardner DK (1998) Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Human Reproduction* **13** 3441–3448.

Elmes M, Green LR, Poore K, Newman J, Burrage D, Abayasekara DRE, Cheng Z, Hanson MA and Wathes DC (2005) Raised dietary n-6 polyunsaturated fatty acid intake increases 2-series prostaglandin production during labour in the ewe. *The Journal of Physiology* **562** 583–592.

Engelhardt H, Tekpetey FR, Gore-Langton RE and Armstrong DT (1992) Regulation of steroid production in cultured porcine thecal cells by transforming growth factor-β. *Molecular and Cellular Endocrinology* **85** 117–126.

Eppig JJ (1981) Prostaglandin E2 stimulates cumulus expansion and hyaluronic acid synthesis by cumuli oophori isolated from mice. *Biology of Reproduction* **25** 191–195.

Espey LL (1980) Ovulation as an inflammatory reaction—A hypothesis. *Biology of Reproduction* **22** 73–106.

Espey LL (1994) Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biology of Reproduction* **50** 233–238.

Estellés A, Cano A, Falcó C, España F, Gilabert J, Grancha S and Aznar J (1999) Lipoprotein(a) levels and isoforms and fibrinolytic activity in postmenopause - Influence of hormone replacement therapy. *Thrombosis and Haemostasis* **81** 104–110.

Estes SJ, Ye B, Qiu W, Cramer D, Hornstein MD and Missmer SA (2009) A proteomic analysis of IVF follicular fluid in women <32 years old. *Fertility and Sterility* **92** 1569–1578.

Fahiminiya S, Reynaud K, Labas V, Batard S, Chastant-Maillard S and Gérard N (2010) Steroid hormones content and proteomic analysis of canine follicular fluid during the preovulatory period. *Reproductive Biology and Endocrinology* **8** 1–14.

Fahiminiya S, Labas V, Roche S, Dacheux J and Gérard N (2011a) Proteomic analysis of mare follicular fluid during late follicle development. *Proteome Science* **9** 1–19.

Fahiminiya S, Labas V, Dacheux JL and Gérard N (2011b) Improvement of 2D-PAGE resolution of human, porcine and equine follicular fluid by means of hexapeptide ligand library. *Reproduction in Domestic Animals* **46** 561–563.

Fahiminiya S, Roche S and Gérard N (2012) Improvement of 2D-PAGE resolution of human, porcine and canine follicular fluid: comparison of two immunodepletion columns. *Reproduction in Domestic Animals = Zuchthygiene* **47** 67–70.

Familiari G, Nottola SA and Motta PM (1986) Focal cell contacts detected by ruthenium red, triton X100 and saponin in the granulosa cells of mouse ovary. *Tissue and Cell* **19** 207–215.

Ferguson EM, Ashworth CJ, Edwards SA, Hawkins N, Hepburn N and Hunter MG (2003) Effect of different nutritional regimens before ovulation on plasma concentrations of metabolic and reproductive hormones and oocyte maturation in gilts. *Reproduction* **126** 61–71.

Ferguson EM, Ashworth CJ, Edwards SA, Hawkins N, Hepburn N and Hunter MG (2004) The effect of feeding a high fibre diet fom mid lactation until breeding on subsequent litter size of sows. In *The Appliance of Pig Science*, 24th ed, pp 175–179. Eds JE Thompson, BP Gill and MA Varley. Nottingham University Press, Nottingham, UK.

Ferguson EM, Slevin J, Edwards SA, Hunter MG and Ashworth CJ (2006) Effect of alterations in the quantity and composition of the pre-mating diet on embryo

survival and foetal growth in the pig. *Animal Reproduction Science* **96** 89–103.

Ferguson EM, Slevin J, Hunter MG, Edwards SA and Ashworth CJ (2007) Beneficial effects of a high fibre diet on oocyte maturity and embryo survival in gilts. *Reproduction* **133** 433–439.

Fernández-Suárez ME, Escolà-Gil JC, Pastor O, Dávalos A, Blanco-Vaca F, Lasunción MA, Martínez-Botas J and Gómez-Coronado D (2016) Clinically used selective estrogen receptor modulators affect different steps of macrophage-specific reverse cholesterol transport. *Scientific Reports* **6** 1–14.

Ferrazza R de A, Schmidt EM dos S, Carmichael MM, Souza FF de, Burchmore R, Sartori R, Eckersall PD and Ferreira JCP (2017) Quantitative proteomic profiling of bovine follicular fluid during follicle development. *Biology of Reproduction* **Nov 17**.

Fiehn O (2016) Metabolomics by gas chromatography-mass spectrometry: the combination of targeted and untargeted profiling. *Current Protocols in Molecular Biology* **144** 30.4.1–30.4.32.

Fleming AD and McGaughey RW (1982) A progesterone binding component in porcine ovarian follicular fluid. *Journal of Endocrinology* **94** 69–76.

Fonslow BR, Stein BD, Webb KJ, Xu T, Choi J, Park SK, Yates JR and Iii JRY (2013) Digestion and depletion of abundant proteins improves proteomic coverage. *Nature Methods* **10** 54–56.

Forde N, Gorman AO, Whelan H, Duffy P, Hara LO, Kelly AK, Havlicek V, Besenfelder U, Brennan L and Lonergan P (2016) Lactation-induced changes in metabolic status and follicular-fluid metabolomic profile in postpartum dairy cows. *Reproduction, Fertility and Development* **28** 1882–1892.

Fu Q, Huang Y, Wang Z, Chen F, Huang D, Lu Y, Liang X and Zhang M (2016) Proteome profile and quantitative proteomic analysis of buffalo (Bubalusbubalis) follicular fluid during follicle development. *International Journal of Molecular Sciences* **17** 1–20.

Funahashi H, Koike T and Sakai R (2008) Effect of glucose and pyruvate on nuclear and cytoplasmic maturation of porcine oocytes in a chemically defined medium. *Theriogenology* **70** 1041–1047.

Geary B, Young IS, Cash P, Whitfield PD and Doherty MK (2016) Comparing simplification strategies for the skeletal muscle proteome. *Proteomes* **4** 1–12.

Geisert RD, Johnson GA and Burghardt RC (2015) Implantation and establishment of pregnacy in the pig. In *Regulation of Implantation and Establishment of Pregnancy in Mammals*, pp 137–164.

Gelissen IC, Hochgrebe T, Wilson MR, Easterbrook-smith SB, Jessup W, Dean RT and Brown AJ (1998) Apolipoprotein J (clusterin) induces cholesterol

export from macrophage-foam cells: a potential anti-atherogenic function? *Biochemical Journal* **331** 231–237.

Genlin W, Jie C, Weihua C and Zhaoyu H (1998) Comparative studies on the concentrations of GnRH, LH, inhibin in serum and inhibin and gonadal steroids in follicular fluids of Erhualian and Large White gilts. *Reproduction in Domestic Animals* **33** 379–381.

Gérard N, Fahiminiya S, Grupen CG and Desbarats L (2015) Reproductive physiology and ovarian folliculogenesis examined via 1H-NMR metabolomics signatures: a Comparative study of large and small follicles in three mammalian species (Bos taurus, Sus scrofa domesticus and Equus ferus caballus). *Omics: A Journal of Integrative Biology* **19** 31–40.

Gika H and Theodoridis G (2011) Sample preparation prior to the LC–MS-based metabolomics/metabonomics of blood-derived samples. *Bioanalysis* **3** 1647–1661.

Di Girolamo F, Righetti PG, Soste M, Feng Y and Picotti P (2013) Reproducibility of combinatorial peptide ligand libraries for proteome capture evaluated by selected reaction monitoring. *Journal of Proteomics* **89** 215–226.

Glasgow VL, Edwards SA, McEvoy TG and Shanks M (1999) The effect of feeding a diet high in fibre during the rearing period on reproduction in gilts. In *Proceedings of the British Society of Animal Science*, p 67.

Goldstein IM, Kaplan HB, Edelson HS and Weissmann G (1982) Ceruloplasmin: An acute phase reactant that scavenges oxygen-derived free radicals. *Annals of the New York Academy of Sciences* **389** 368–379.

González-Serrano AF, Ferreira CR, Pirro V, Lucas-Hahn A, Heinzmann J, Hadeler K-G, Baulain U, Aldag P, Meyer U, Piechotta M *et al.* (2015) Effects of long-term dietary supplementation with conjugated linoleic acid on bovine oocyte lipid profile. *Reproduction, Fertility and Development* **28** 1326–1339.

Gregoraszczuk EL, Ptak A, Wojtowicz AK, Gorska T and Nowak KW (2004) Estrus cycle-dependent action of leptin on basal and GH or IGF-I stimulated steroid secretion by whole porcine follicles. *Endocrine Regulations* **38** 15–21.

Grupen CG and Armstrong DT (2010) Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. *Reproduction, Fertility and Development* **22** 1100–1109.

Grupen CG, McIlfatrick SM, Ashman RJ, Boquest AC, Armstrong DT and Nottle MB (2003) Relationship between donor animal age, follicular fluid steroid content and oocyte developmental competence in the pig. *Reproduction, Fertility and Development* **15** 81–87.

Guillemet R, Hamard A, Quesnel H, Père MC, Etienne M, Dourmad JY and

Meunier-Salaün MC (2007) Dietary fibre for gestating sows: effects on parturition progress, behaviour, litter and sow performance. *Animal* **1** 872–880.

Guzmán P, Saldaña B, Bouali O, Cámara L and Mateos GG (2016) Effect of level of fiber of the rearing phase diets on egg production, digestive tract traits, and body measurements of brown egg-laying hens fed diets differing in energy concentration. *Poultry Science* **95** 1836–1847.

Hammond JM, Hsu CJ, Klindt J, Tsang BK and Downey BR (1988) Gonadotropins increase concentrations of immunoreactive insulin-like growth factor-I in porcine follicular fluid in vivo. *Biology of Reproduction* **38** 304–308.

Han J, Lee H-D, Bibbs L and Ulevitch R (1994) A MAP Kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265** 808–811.

Hanczakowska E and Świątkiewicz M (2013) Legume seeds and rapeseed press cake as substitutes for soybean meal in sow feed. *Annals of Animal Science* **22** 435–444.

Hanrieder JJ, Nyakas A, Naessén T and Bergquist J (2008) Proteomic analysis of human follicular fluid using an alternative bottom-up approach. *Journal of Proteome Research* **7** 443–449.

Hanrieder J, Zuberovic A and Bergquist J (2009) Surface modified capillary electrophoresis combined with in solution isoelectric focusing and MALDI-TOF/TOF MS: A gel-free multidimensional electrophoresis approach for proteomic profiling - Exemplified on human follicular fluid. *Journal of Chromatography A* **1216** 3621–3628.

Hashemitabar M, Bahmanzadeh M, Mostafaie A, Orazizadeh M, Farimani M and Nikbakht R (2014) A proteomic analysis of human follicular fluid: Comparison between younger and older women with normal FSH levels. *International Journal of Molecular Sciences* **15** 17518–17540.

Hatada EN, Krappmann D and Scheidereit C (2000) NF-κB and the innate immune response. *Current Opinion in Immunology* **12** 52–58.

Hatanaka E, Dermargos A, Hirata AE, Vinolo MAR, Carpinelli AR, Newsholme P, Armelin HA and Curi R (2013) Oleic, linoleic and linolenic acids increase ROS production by fibroblasts via NADPH oxidase activation. *PLOS ONE* **8** e58626.

Hayes E, Kushnir V, Ma X, Biswas A, Prizant H, Gleicher N and Sen A (2016) Intra-cellular mechanism of Anti-Müllerian hormone (AMH) in regulation of follicular development. *Molecular and Cellular Endocrinology* **433** 56–65.

Hedemann MS, Theil PK, Lærke HN and Bach Knudsen KE (2015) Distinct difference in absorption pattern in pigs of betaine provided as a supplement or present naturally in cereal dietary fiber. *Journal of Agricultural and Food Chemistry* **63** 2725–2733.

Herrick JR, Brad AM and Krisher RL (2006) Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation in vitro. *Reproduction* **131** 289–298.

Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E and Lippman M (1997) A maternal diet high in n-6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. *Proceedings of the National Academy of Sciences* **94** 9372–9377.

Holt JP, Johnston LJ, Baidoo SK and Shurson GC (2006) Effects of a high-fiber diet and frequent feeding on behavior, reproductive performance, and nutrient digestibility in gestating sows. *Journal of Animal Science* **84** 946–955.

Homa ST and Brown C a (1992) Changes in linoleic acid during follicular development and inhibition of spontaneous breakdown of germinal vesicles in cumulus-free bovine oocytes. *Journal of Reproduction and Fertility* **94** 153–160.

Hong J and Lee E (2007) Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development. *Theriogenology* **68** 728–735.

Hong JY, Yong HY, Lee BC, Hwang WS, Lim JM and Lee ES (2004) Effects of amino acids on maturation, fertilization and embryo development of pig follicular oocytes in two IVM media. *Theriogenology* **62** 1473–1482.

Horie K, Takakura K, Taii S, Narimoto K, Noda Y, Nishikawa S, Nakayama H, Fujita J and Mori T (1991) The expression of c-kit protein during oogenesis and early embryonic development. *Biology of Reproduction* **45** 547–552.

Hughes J, Kwong WY, Li D, Salter AM, Lea RG and Sinclair KD (2011) Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. *Reproduction* **141** 105–118.

Hunter RH and Poyser NL (1985) Ovarian follicular fluid concentrations of prostaglandins E2, F2 alpha and I2 during the pre-ovulatory period in pigs. *Reproduction, Nutrition, Developement* **25** 909–917.

van den Hurk R and van de Pavert SA (2001) Localization of an activin/activin receptor system in the porcine ovary. *Molecular Reproduction and Development* **60** 463–471.

Illera MJ, Lorenzo PL, Illera JC and Petters RM (1998) Developmental competence of immature pig oocytes under the influence of EGF, IGF-I, follicular fluid and gonadotropins during IVM-IVF processes. *The International Journal of Developmental Biology* **42** 1169–1172.

Ismail RS, Okawara Y, Fryer JN and Vanderhyden BC (1996) Hormonal regulation of the ligand for c-kit in the rat ovary and its effects on spontaneous oocyte meiotic maturation. *Molecular Reproduction and Development* **43** 458–

469.

Jarkovska K, Martinkova J, Liskova L, Halada P, Moos J, Rezabek K, Gadher SJ and Kovarova H (2010) Proteome mining of human follicular fluid reveals a crucial role of complement cascade and key biological pathways in women undergoing in vitro fertilization. *Journal of Proteome Research* **9** 1289–1301.

Jarkovska K, Skalnikova HK, Halada P, Hrabakova R, Moos J, Rezabek K, Gadher SJ and Kovarova H (2011) Development of ovarian hyperstimulation syndrome: interrogation of key proteins and biological processes in human follicular fluid of women undergoing in vitro fertilization. *Molecular Human Reproduction* **17** 679–692.

Jarrett S and Ashworth CJ (2018) The role of dietary fibre in pig production, with a particular emphasis on reproduction. *Journal of Animal Science and Biotechnology* **9** 59.

Jatesada J, Elisabeth P and Anne-Marie D (2013) Seminal plasma did not influence the presence of transforming growth factor-β1, interleukine-10 and interleukin-6 in porcine follicles shortly after insemination. *Acta Veterinaria Scandinavica* **55** 1–7.

Jégou M, Gondret F, Lalande-Martin J, Tea I, Baéza E and Louveau I (2016) NMR-based metabolomics highlights differences in plasma metabolites in pigs exhibiting diet-induced differences in adiposity. *European Journal of Nutrition* **55** 1189–1199.

Jenne DE, Lowin B, Peitsch MC, Bottchers A, Schmitzg G and Tschopp J (1991) Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. *The Journal of Biological Chemistry* **266** 11030–11036.

De Jesus RJ, Galazzi RM, De Lima TB, Banzato CEM, De Almeida Lima e Silva FL, Dantas CDR, Gozzo CF and Arruda MAZ (2017a) Simplifying the human serum proteome for discriminating patients with bipolar disorder of other psychiatry conditions. *Clinical Biochemistry* **50** 1118–1125.

De Jesus RJ, Fernandes R da S, De Souza Pessoa G, Raimundo Jr IM and Arruda MAZ (2017b) Depleting high-abundant and enriching low-abundant proteins in human serum: An evaluation of sample preparation methods using magnetic nanoparticle, chemical depletion and immunoaffinity techniques. *Talanta* **170** 199–209.

Jia B, Wu G, Fu X, Mo X, Du M, Hou Y and Zhu S (2014) Trans-10, cis -12 conjugated linoleic acid enhances in vitro maturation of porcine oocytes. *Molecular Reproduction and Development* **81** 20–30.

John GB, Shidler MJ, Besmer P and Castrillon DH (2009) Kit Signaling via PI3K promotes ovarian follicle maturation but is dispensable for primordial follicle activation. *Developmental Biology* **331** 292–299.

Johnson MH (2007) Ovarian function in the adult. In *Essential Reproduction*, pp 80–102.

Johnson KA and Goody RS (2011) The original Michaelis constant: Translation of the 1913 Michaelis-Menten paper. *Biochemistry* **50** 8264–8269.

Jorritsma R, César ML, Hermans JT, Kruiwagen CLJJ, Vos PLAM and Kruip TAM (2004) Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Animal Reproduction Science* **81** 225–235.

Juchem SO, Cerri RLA, Villaseñor M, Galváo KN, Bruno RGS, Rutigliano HM, DePeters EJ, Silvestre FT, Thatcher WW and Santos JEP (2010) Supplementation with calcium salts of linoleic and trans-octadecenoic acids improves fertility of lactating dairy cows. *Reproduction in Domestic Animals* **45** 55–62.

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH (2011) Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertility and Sterility* **95** 1970–1974.

Ka H-H, Sawai K, Wang W-H, Im K-S and Niwa K (1997) Amino acids in maturation medium and presence of cumulus cells at fertilization promote male pronuclear formation in porcine oocytes matured and penetrated in vitro. *Biology of Reproduction* **57** 1478–1483.

Kalaany NY and Mangelsdorf DJ (2006) LXRs AND FXR: The Yin and Yang of cholesterol and fat metabolism. *Annual Review of Physiology* **68** 159–191.

Kawashima I, Okazaki T, Noma N, Nishibori M, Yamashita Y and Shimada M (2008) Sequential exposure of porcine cumulus cells to FSH and/or LH is critical for appropriate expression of steroidogenic and ovulation-related genes that impact oocyte maturation in vivo and in vitro. *Reproduction* **136** 9–21.

Kazemi A, Ramezanzadeh F, Nasr-Esfahani MH, Yaraghi AS and Ahmadi M (2013) Does dietary fat intake influence oocyte competence and embryo quality by inducing oxidative stress in follicular fluid? *Iran Journal of Reproductive Medicine* **11** 1005–1012.

Kempisty B, Antosik P, Bukowska D, Jackowska M, Lianeri M, Jaśkowski J and Jagodziński P (2008) Analysis of selected transcript levels in porcine spermatozoa, oocytes, zygotes and two-cell stage embryos. *Reproduction, Fertility and Development* **20** 513–518.

Khalil WA, Marei WFA and Khalid M (2013) Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. *Theriogenology* **80** 161–168.

Khandoker MAMY, Tsujii H and Karasawa D (1997) Fatty acid compositions of

oocytes, follicular, oviductal and uterine fluids of pig and cow. *Asian-Australasian Journal of Animal Sciences* **10** 523–527.

Kidder GM and Vanderhyden BC (2010) Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence Gerald. *The Canadian Journal of Physiology and Pharmacology* **88** 399–413.

Kihara T, Kimura A, Moriyama A, Ohkubo I and Takahashi T (2000) Identification of components of the intrafollicular bradykinin-producing system in the porcine ovary. *Biology of Reproduction* **62** 1160–1167.

Kim Y-SS, Kim M-SS, Lee S-HH, Choi B-CC, Lim J-MM, Cha KY and Baek K-HH (2006) Proteomic analysis of recurrent spontaneous abortion: Identification of an inadequately expressed set of proteins in human follicular fluid. *Proteomics* **6** 3445–3454.

Kim Y, Gu B, Choi B, Kim M, Song S, Yun J, Chung MIK, Choi C and Baek K (2013) Apolipoprotein A-IV as a novel gene associated with polycystic ovary syndrome. *International Journal of Molecular Medicine* **31** 707–716.

Kimura A, Kihara T, Okimura H, Hamabata T, Ohnishi J, Moriyama A, Takahashi K and Takahashi T (2000) Identification of porcine follipsin as plasma kallikrein, and its possible involvement in the production of bradykinin within the follicles of porcine ovaries. *Molecular Reproduction and Development* **57** 79–87.

Kling OR, Roche PC, Campeau JD, Nishimura K, Nakamura RM and DiZerega GS (1984) Identification of a porcine follicular fluid fraction which suppresses follicular response to gonadotropins. *Biology of Reproduction* **30** 564–572.

Knight PG, Satchell L and Glister C (2012) Intra-ovarian roles of activins and inhibins. *Molecular and Cellular Endocrinology* **359** 53–65.

Knox R V., Vatzias G, Naber CH and Zimmerman DR (2003) Plasma gonadotropins and ovarian hormones during the estrous cycle in high compared to low ovulation rate gilts. *Journal of Animal Science* **81** 249–260.

Knudsen JF, Litkowski LJ, Wilson TL, Guthrie HD and Batta SK (1978) Concentrations of hydrogen ions, oxygen, carbon dioxide and bicarbonate in porcine follicular fluid. *Journal of Endocrinology* **79** 249–250.

Koketsu Y, Tani S and Iida R (2017) Factors for improving reproductive performance of sows and herd productivity in commercial breeding herds. *Porcine Health Management* **3** 1.

Krause BR, Sloop CH, Castle CK and Roheim PS (1981) Mesenteric lymph apolipoproteins in control and ethinyl estradiol-treated rats: a model for studying apolipoproteins of intestinal origin. *Journal of Lipid Research* **22** 610–619.

Krisher RL (2013) In vivo and in vitro environmental effects on mammalian oocyte

quality. *Annual Review of Animal Biosciences* **1** 393–417.

Kristensen M and Bügel S (2011) A diet rich in oat bran improves blood lipids and hemostatic factors, and reduces apparent energy digestibility in young healthy volunteers. *European Journal of Clinical Nutrition* **65** 1053–1058.

Kushner I (1982) The phenomenon of the acute phase response. *Annals New York Academy of Sciences* **389** 39–48.

Kushnir MM, Naessén T, Wanggren K, Rockwood AL, Crockett DK and Bergquist J (2012) Protein and steroid profiles in follicular fluid after ovarian hyperstimulation as potential biomarkers of IVF outcome. *Journal of Proteome Research* **11** 5090–5100.

Kyriazakis I and Whittemore CT (2006) Chapter 4: Reproduction. In *Whittemore's Science and Practice of Pig Production*, Third Edit, pp 104–147. Oxford, UK: Blackwell Publishing.

Laitinen M, Rutanen EM and Ritvos O (1995) Expression of c-kit ligand messenger ribonucleic acids in human ovaries and regulation of their steady state levels by gonadotropins in cultured granulosa-luteal cells. *Endocrinology* **136** 4407–4414.

Lapa M, Marques CC, Alves SP, Vasques MI, Baptista MC, Carvalhais I, Pereira MS, Horta AEM, Bessa RJB and Pereira R (2011) Effect of trans-10 cis-12 conjugated linoleic acid on bovine oocyte competence and fatty acid composition. *Reproduction in Domestic Animals* **46** 904–910.

Lee GR, Nacht S, Lukens JN and Cartwmgwr GE (1968) Iron metabolism in copper-eficient swine. *The Journal of Clinical Investigation* **47** 2058–2069.

Lee H-C, Lee S-W, Lee KW, Lee S-W, Cha K-Y, Kim KH and Lee S (2005) Identification of new proteins in follicular fluid from mature human follicles by direct sample rehydration method of two-dimensional polyacrylamide gel electrophoresis. *Journal of Korean Medical Science* **20** 456–460.

Lee TW, Tumanov S, Villas-Bôas SG, Montgomery JM and Birch NP (2015) Chemicals eluting from disposable plastic syringes and syringe filters alter neurite growth, axogenesis and the microtubule cytoskeleton in cultured hippocampal neurons. *Journal of Neurochemistry* **133** 53–65.

Van Leeuwen MA and Van Rijswijk MH (1994) Acute phase proteins in the monitoring of inflammatory disorders. *Baillière's Clinical Rheumatology* **8** 531–552.

Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Soom A Van, Bols PEJ and Kruif A De (2004) Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. *Animal Reproduction Science* **80** 201–211.

Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A,

Genicot G and Van Soom A (2005) Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* **130** 485–495.

Levenson AS, Kwaan HC, Svoboda KM, Weiss IM, Sakurai S and Jordan VC (1998) Oestradiol regulation of the components of the plasminogen-plasmin system in MDA-MB-231 human breast cancer cells stably expressing the oestrogen receptor. *British Journal of Cancer* **78** 88–95.

Li L, Xu Y and Yu C (2012a) Proteomic analysis of serum of women with elevated Ca-125 to differentiate malignant from benign ovarian tumors. *Asian Pacific Journal of Cancer Prevention* **13** 3265–3270.

Li K, He N, Yuan H, Wang J, Sui H and Tan J (2012b) Characterization of cumulus expansion-inhibiting factor (CEIF) in goat follicles. *Biology of Reproduction* **86** 1–9.

Li Y, Zhang ZZ, He CJ, Zhu KF, Xu ZY, Ma T, Tao JL and Liu GS (2015) Melatonin protects porcine oocyte in vitro maturation from heat stress. *Journal of Pineal Research* **59** 365–375.

Lim H-J, Seok AE, Han J, Lee J, Lee S, Kang H-G, Cha BH and Yang Y (2017) N-glycoproteomic analysis of human follicular fluid during natural and stimulated cycles in patients undergoing in vitro fertilization. *Clinical and Experimental Reproductive Medicine* **44** 63–72.

Lippolis JD and Reinhardt TA (2010) Utility, limitations, and promise of proteomics in animal science. *Veterinary Immunology and Immunopathology* **138** 241–251.

Liu R-H, Li Y-H, Jiao L-H, Wang X-N, Wang H and Wang W-H (2002) Extracellular and intracellular factors affecting nuclear and cytoplasmic maturation of porcine oocytes collected from different sizes of follicles. *Zygote* **10** 253–260.

Loisel F, Farmer C, Ramaekers P and Quesnel H (2013) Effects of high fiber intake during late pregnancy on sow physiology, colostrum production, and piglet performance. *Journal of Animal Science* **91** 5269–5279.

Longo V, Rebulla P, Pupella S, Zolla L and Rinalducci S (2016) Proteomic characterization of platelet gel releasate from adult peripheral and cord blood. *Proteomics. Clinical Applications* **10** 870–882.

Lorkova L, Pospisilova J, Lacheta J, Leahomschi S, Zivny J, Cibula D, Zivny J and Petrak J (2012) Decreased concentrations of retinol-binding protein 4 in sera of epithelial ovarian cancer patients: A potential biomarker identified by proteomics. *Oncology Reports* **27** 318–324.

Lössl P, van de Waterbeemd M and Heck AJR (2016) The diverse and expanding role of mass spectrometry in structural and molecular biology. *The EMBO*

Journal **35** 2634–2657.

Lui L, Dai Y and Moor RM (1997) Role of secreted proteins and gonadotrophins in promoting full maturation of porcine oocytes in vitro. *Molecular Reproduction and Development* **47** 191–199.

Maeda A, Goto Y, Matsuda-Minehata F, Cheng Y, Inoue N and Manabe N (2007a) Changes in expression of interleukin-6 receptors in granulosa cells during follicular atresia in pig ovaries. *Journal of Reproduction and Development* **53** 727–736.

Maeda A, Inoue N, Matsuda-Minehata F, Goto Y, Cheng Y and Manabe N (2007b) The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. *Journal of Reproduction and Development* **53** 481–490.

Magnani B, Tsen L, Datta S and Bader A (1999) In vitro fertilization: do short-term changes in estrogen levels produce increased fibrinolysis? *American Journal of Clinical Pathology* **112** 485–491.

Mahajan DK and Little AB (1978) Specific cortisol binding protein porcine follicular fluid. *Biology of Reproduction* **18** 834–842.

Mahajan DK, Billiar RB and Little AB (1980) Isolation of cortisol binding globulin (CBG) from porcine follicular fluid by affinity chromatography. *Journal of Steroid Biochemistry* **13** 67–71.

Manfredi M, Brandi J, Conte E, Pidutti P, Gosetti F, Robotti E, Marengo E and Cecconi D (2017) IEF peptide fractionation method combined to shotgun proteomics enhances the exploration of rice milk proteome. *Analytical Biochemistry* **537** 72–77.

Maniwa J, Izumi S, Isobe N and Terada T (2005) Studies on substantially increased proteins in follicular fluid of bovine ovarian follicular cysts using 2-D PAGE and MALDI-TOF MS. *Reproductive Biology and Endocrinology* **3** 1–9.

Marco-Ramell A and Bassols A (2010) Enrichment of low-abundance proteins from bovine and porcine serum samples for proteomic studies. *Research in Veterinary Science* **89** 340–343.

Marei WF, Wathes DC and Fouladi-Nashta AA (2010) Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction* **139** 979–988.

Marei WF, Wathes DC and Fouladi-Nashta AA (2012) Differential effects of linoleic and alpha-linolenic fatty acids on spatial and temporal mitochondrial distribution and activity in bovine oocytes. *Reproduction, Fertility and Development* **24** 679–690.

Martínez-Paredes E, Ródenas L, Martínez-Vallespín B, Cervera C, Blas E, Brecchia G, Boiti C and Pascual JJ (2012) Effects of feeding programme on

the performance and energy balance of nulliparous rabbit does. *Animal* **6** 1086–1095.

Martinez-Puig D, Pérez JF, Castillo M, Andaluz A, Anguita M, Morales J and Gasa J (2003) Consumption of raw potato starch increases colon length and fecal excretion of purine bases in growing pigs. *Journal of Nutrition* **133** 134–139.

Matoba S, Bender K, Fahey AG, Mamo S, Brennan L, Lonergan P and Fair T (2014) Predictive value of bovine follicular components as markers of oocyte developmental potential. *Reproduction, Fertility and Development* **26** 337–345.

Matsumoto D and Chamley WA (1980) Identification of relaxins in porcine follicular fluid and in the ovary of the immature sow. *Journal of Reproduction and Fertility* **58** 369–375.

Matte JJ, Robert S, Girard CL, Farmer C and Martineau GP (1994) Effect of bulky diets based on wheat bran or oat hulls on reproductive performance of sows during their first two parities. *Journal of Animal Science* **72** 1754–1760.

Mattioli M, Bacci ML, Galeati G and Seren E (1989) Developmental competence of pig oocytes matured and fertilized in vitro. *Theriogenology* **31** 1201–1207.

Matukumalli SR, Tangirala R and Rao CM (2017) Clusterin: full-length protein and one of its chains show opposing effects on cellular lipid accumulation. *Scientific Reports* **7** 1–13.

McCoard SA, Wise TH and Ford JJ (2002) Expression levels of Mullerian-inhibiting substance, GATA4 and 17α-hydroxylase/17,20-lyase cytochrome P450 during embryonic gonadal development in two diverse breeds of swine. *Journal of Endocrinology* **175** 365–374.

Mcdonald JK (1990) Role of neuropeptide Y in reproductive function. *Annals of the New York Academy of Sciences* **611** 258–272.

McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JSM and Speake BK (2000) Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *Journal of Reproduction and Fertility* **118** 163–170.

McGlone JJ and Fullwood SD (2001) Behavior, reproduction, and immunity of crated pregnant gilts: Effects of high dietary fiber and rearing environment. *Journal of Animal Science* **79** 1466–1474.

Mcrae C, Sharma V and Fisher J (2013) Metabolite profiling in the pursuit of biomarkers for IVF outcome: The case for metabolomics studies. *International Journal of Reproductive Medicine* **2013** 603167.

McRae C, Baskind NE, Orsi NM, Sharma V and Fisher J (2012) Metabolic profiling of follicular fluid and plasma from natural cycle in vitro fertilization patients- a pilot study. *Fertility and Sterility* **98** 1449–1457.

Miller S, Batra A, Shearrer G, House B, Cook L, Pont S, Goran M and Davis J (2015) Dietary fibre linked to decreased inflammation in overweight minority youth. *Pediatric Obesity* **11** 33–39.

Mirabi P, Chaichi MJ, Esmaeilzadeh S, Jorsaraei GSA, Bijani A, Mahjoobeh E and Karooee SFH (2017a) The role of fatty acids on ICSI outcomes: a prospective cohort study. *Lipids in Health and Disease* **16** 1–9.

Mirabi P, Javad M, Esmaeilzadeh S, Jorsaraei SGA, Bijani A and Ehsani M (2017b) Does different BMI influence oocyte and embryo quality by inducing fatty acid in follicular fluid? *Taiwanese Journal of Obstetrics & Gynecology* **56** 159–164.

Mittal RD (2015) Tandem mass spectroscopy in diagnosis and clinical research. *Indian Journal of Clinical Biochemistry* **30** 121–123.

Mollace V, Muscoli C, Masini E, Cuzzocrea S and Salvemini D (2005) Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacological Reviews* **57** 217–252.

Moniruzzaman M and Miyano T (2007) KIT-KIT ligand in the growth of porcine oocytes in primordial follicles. *The Journal of Reproduction and Development* **53** 1273–1281.

Montiel JF, Kaune H and Maliqueo M (2013) Maternal-fetal unit interactions and eutherian neocortical development and evolution. *Frontiers in Neuroanatomy* **7** 1–14.

Moore L, Knapp HR and Landon EJ (1977) An effect of estradiol and testosterone on the calcium pump activity and phospholipid fatty acid composition of rat liver microsomes. *Endocrinology* **100** 1516–1520.

Mosesson MW (2005) Fibrinogen and fibrin structure and functions. *Journal of Thrombosis and Haemostasis* **3** 1894–1904.

Motro B and Bernstein A (1993) Dynamic changes in ovarian c- kit and Steel expression during the estrous reproductive cycle. *Developmental Dynamics* **197** 69–79.

Mouzat K, Volat F, Baron S, Alves G, Pommier AJC, Volle DH, Marceau G, DeHaze A, Déchelotte P, Duggavathi R *et al.* (2009) Absence of nuclear receptors for oxysterols liver X receptor induces ovarian hyperstimulation syndrome in mice. *Endocrinology* **150** 3369–3375.

Naessen T, Kushnir MM, Chaika A, Nosenko J, Mogilevkina I, Rockwood AL, Carlstrom K, Bergquist J and Kirilovas D (2010) Steroid profiles in ovarian follicular fluid in women with and without polycystic ovary syndrome, analyzed by liquid chromatography-tandem mass spectrometry. *Fertility and Sterility* **94** 2228–2233.

Nagyova E (2012) Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation. *Endocrine Regulations* **46** 225–235.

Nagyova E, Camaioni A, Prochazka R and Salustri A (2004) Covalent transfer of heavy chains of inter-α-trypsin inhibitor family proteins to hyaluronan in in vivo and in vitro expanded porcine oocyte-cumulus complexes. *Biology of Reproduction* **71** 1838–1843.

Nagyová E, Procházka R and Motlík J (1997) Porcine oocytes produce CEEF only during their growth period and transition to metaphase I. *Theriogenology* **47** 197.

Nakano M, Kato Y and Tsunoda Y (2017) Effect of melatonin treatment on the developmental potential of parthenogenetic and somatic cell nuclear-transferred porcine oocytes in vitro. *Zygote* **20** 199–207.

Nevoral J, Orsák M, Klein P, Petr J, Dvořáková M, Weingartová I, Vyskočilová A, Zámostná K, Krejčová T and Jílek F (2014) Cumulus cell expansion, its role in oocyte biology and perspectives of measurement: A Review. *Scientia Agriculturae Bohemica* **45** 212–225.

Nilsson EE and Skinner MK (2003) Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction* **69** 1265–1272.

Nilsson EE and Skinner MK (2004) Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Molecular and Cellular Endocrinology* **214** 19–25.

Nilsson EE, Kezele P and Skinner MK (2002) Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Molecular and Cellular Endocrinology* **188** 65–73.

Nilsson E, Rogers N and Skinner MK (2007) Actions of anti-Müllerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. *Reproduction* **134** 209–221.

Niu Z, Lin N, Gu R, Sun Y and Feng Y (2014) Associations between insulin resistance, free fatty acids, and oocyte quality in polycystic ovary syndrome during in vitro fertilization. *Journal of Clinical Endocrinology and Metabolism* **99** E2269–E2276.

Noblet J and Le Goff G (2001) Effect of dietary fibre on the energy value of feeds for pigs. *Animal Feed Science and Technology* **90** 35–52.

Ny A, Nordström L, Carmeliet P and Ny T (1997) Studies of mice lacking plasminogen activator gene function suggest that plasmin production prior to ovulation exceeds the amount needed for optimal ovulation efficiency. *European Journal of Biochemistry* **244** 487–493.

O'Doherty AM, O'Gorman A, Naib A al, Brennan L, Daly E, Duffy P and Fair T (2014) Negative energy balance affects imprint stability in oocytes recovered from postpartum dairy cows. *Genomics* **104** 177–185.

O'Gorman A, Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M and Brennan L (2013) Metabolic profiling of human follicular fluid identifies potential biomarkers of oocyte developmental competence. *Reproduction* **146** 389–395.

Ogiwara K, Minagawa K, Takano N, Kageyama T and Takahashi T (2012) Apparent involvement of plasmin in early-stage follicle rupture during ovulation in medaka. *Biology of Reproduction* **86** 1–10.

Oh JW, Kim SK, Cho K-C, Kim M-S, Suh CS, Lee JR and Kim KP (2017) Proteomic analysis of human follicular fluid in poor ovarian responders during in vitro fertilization. *Proteomics* **17** 1–11.

Oliviero C, Kokkonen T, Heinonen M, Sankari S and Peltoniemi O (2009) Feeding sows with high fibre diet around farrowing and early lactation: Impact on intestinal activity, energy balance related parameters and litter performance. *Research in Veterinary Science* **86** 314–319.

Opalka M, Dusza L, Koziorowski M, Staszkiewicz J, Lipiński K and Tywoñczuk J (2001) Effect of long-term feeding with graded levels of low glucosinolate rapeseed meal on endocrine status of gilts and their piglets. *Livestock Production Science* **69** 233–243.

Orvieto R, Chen R, Ashkenazi J, Ben-Haroush A, Bar J and Fisch B (2004) C-reactive protein levels in patients undergoing controlled ovarian hyperstimulation for IVF cycle. *Human Reproduction* **19** 357–359.

Ostermeier GC, Miller D, Huntriss JD, Diamond MP and Krawetz SA (2004) Delivering spermatozoan RNA to the oocyte. *Nature* **429** 154.

Oxender D, Colenbrander B, van deWiel DFM and G WCJ (1979) Ovarian development in fetal and prepubertal pigs. *Biology of Reproduction* **21** 715–721.

Paczkowski M, Silva E, Schoolcraft WB and Krisher RL (2013) Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes. *Biology of Reproduction* **88** 1–11.

Palacín M, Estévez R, Bertran J and Zorzano A (1998) Molecular biology of mammalian plasma membrane amino acid transporters. *Physiological Reviews* **78** 969–1054.

Pampfer S, Vanderheyden I, McCracken JE, Vesela J and De Hertogh R (1997) Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-α in vitro. *Development* **124** 4827–4836.

Parrott JA, Whaley PD and Skinner MK (1993) Extrahepatic expression of fibrinogen by granulosa cells: potential role in ovulation. *Endocrinology* **133** 1645–1649.

Paula Junior AR, van Tilburg MF, Lobo MDP, Monteiro-Moreira ACO, Moreira RA, Melo CHS, Souza-Fabjan JMG, Araújo AA, Melo LM, Teixeira DIA *et al.* (2017) Proteomic analysis of follicular fluid from tropically-adapted goats. *Animal Reproduction Science* **S0378-4320** 1–10.

Paulus A, Freeby S, Academia K, Thulasiraman V, Wehr T, Liu N, Roth S and Smith K (2009) Accessing low-abundance proteins in serum and plasma with a novel, simple enrichment and depletion method. *Bio-Rad Sample Preparation* **Tech Note** 1–6.

Pawlak P, Cieslak A, Warzych E, Zejden Z, Szumacher-Strabel M, Molinska-Glura M and Lechniak D (2012) No single way to explain cytoplasmic maturation of oocytes from prepubertal and cyclic gilts. *Theriogenology* **78** 2020–2030.

van der Peet-Schwering CMC, Kemp B, Binnendijk GP, den Hartog LA, Spoolder HAM and Verstegen MWA (2003) Performance of sows fed high levels of nonstarch polysaccharides during gestation and lactation over three parities. *Journal of Animal Science* **81** 2247–2258.

Pekkinen J, Olli K, Huotari A, Tiihonen K, Keski-Rahkonen P, Lehtonen M, Auriola S, Kolehmainen M, Mykkänen H, Poutanen K *et al.* (2013) Betaine supplementation causes increase in carnitine metabolites in the muscle and liver of mice fed a high-fat diet as studied by nontargeted LC-MS metabolomics approach. *Molecular Nutrition and Food Research* **57** 1959–1968.

Peltoniemi OAT, Tast A, Heinonen M, Oravainen J, Munsterhjelm C, Hälli O, Oliviero C, Hämeenoja P and Virolainen J V (2010) Fertility of sows fed ad libitum with a high fibre diet during pregnancy. *Reproduction in Domestic Animals* **45** 1008–1014.

Percy AJ, Chambers AG, Yang J, Hardie DB and Borchers CH (2014) Advances in multiplexed MRM-based protein biomarker quantitation toward clinical utility. *Biochimica et Biophysica Acta* **1844** 917–926.

Perez-Riverol Y, Alpi E, Wang R, Hermjakob H and Vizca JA (2015) Making proteomics data accessible and reusable: Current state of proteomics databases and repositories. *Proteomics* **15** 930–949.

Piñero-Sagredo E, Nunes S, de los Santos MJ, Celda B and Esteve V (2010) NMR metabolic profile of human follicular fluid. *NMR in Biomedicine* **23** 485–495.

Ponter AA, Guyader-Joly C, Nuttinck F, Grimard B and Humblot P (2012) Oocyte and embryo production and quality after OPU-IVF in dairy heifers given diets varying in their n-6/n-3 fatty acid ratio. *Theriogenology* **78** 632–645.

Prates EG, Alves SP, Marques CC, Baptista MC, Horta AEM, Bessa RJB and Pereira RM (2013a) Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators trans -10, cis -12 conjugated linoleic acid (t10, c12 CLA) and forskolin. *In Vitro Cellular and Developmental Biology*. *Animal* **49** 335–345.

Prates EG, Marques CC, Baptista MC, Vasques MI, Carolino N, Horta AEM, Charneca R, Nunes JT and Pereira RM (2013b) Fat area and lipid droplet morphology of porcine oocytes during in vitro maturation with trans-10 , cis-12 conjugated linoleic acid and forskolin. *Animal* **7** 602–609.

Prentice-Biensch JR, Singh J, Alfoteisy B and Anzar M (2012) A simple and high-throughput method to assess maturation status of bovine oocytes: Comparison of anti-lamin A/C-DAPI with an aceto-orcein staining technique. *Theriogenology* **78** 1633–1638.

Price RKR, Keaveney EME, Hamill LL, Wallace JMW, Ward M, Ueland PM, McNulty H, Strain JJ, Parker MJ and Welch RW (2010) Consumption of wheat aleurone-rich foods increases fasting plasma betaine and modestly decreases fasting homocysteine and LDL-cholesterol in adults. *The Journal of Nutrition* **140** 784–790.

Prochazka R, Kalab P and Nagyova E (2003) Epidermal growth factor-receptor tyrosine kinase activity regulates expansion of porcine oocyte-cumulus cell complexes in vitro. *Biology of Reproduction* **68** 797–803.

Prochazka R, Blaha M and Nemcova L (2012) Signaling pathways regulating FSH- and amphiregulin-induced meiotic resumption and cumulus cell expansion in the pig. *Reproduction* **144** 535–546.

Procházka R, Sršeň V, Nagyová E, Miyano T and Flechon JE (2000) Developmental regulation of effect of epidermal growth factor on porcine oocyte-cumulus cell complexes: Nuclear maturation, expansion, and F-actin remodeling. *Molecular Reproduction and Development* **56** 63–73.

Quesnel H, Meunier-Salaün MC, Hamard A, Guillemet R, Etienne M, Farmer C, Dourmad JY and Père MC (2009) Dietary fiber for pregnant sows: Influence on sow physiology and performance during lactation. *Journal of Animal Science* **87** 532–543.

Racowsky C and McGaughey RW (1982) Further studies of the effects of follicular fluid and membrana granulosa cells on the spontaneous maturation of pig oocytes. *Journal of Reproduction and Fertility* **66** 505–512.

Rauniyar N, Peng G, Lam TT, Zhao H, Mor G and Williams KR (2017) Data-independent acquisition and parallel reaction monitoring mass spectrometry identification of serum biomarkers for ovarian cancer. *Biomarker Insights* **12** 1–12.

Regiani T, Cordeiro FB, da Costa L do VT, Salgueiro J, Cardozo K, Carvalho

VM, Perkel KJ, Zylbersztejn DS, Cedenho AP and Lo Turco EG (2015) Follicular fluid alterations in endometriosis: label-free proteomics by MS(E) as a functional tool for endometriosis. *Systems Biology in Reproductive Medicine* **61** 263–276.

Ren K, Tang Z, Jiang Y, Tan Y and Yi G (2015) Apolipoprotein M. *Clinica Chimica Acta* **446** 21–29.

Renaudeau D, Anaïs C and Noblet J (2003) Effects of dietary fiber on performance of multiparous lactating sows in a tropical climate. *Journal of Animal Science* **81** 717–725.

Renaville B, Bacciu N, Comin A, Motta M, Poli I, Vanini G and Prandi A (2010) Plasma and follicular fluid fatty acid profiles in dairy cows. *Reproduction in Domestic Animals* **45** 118–121.

Renteria-Flores JA, Johnston LJ, Shurson GC, Moser RL and Webel SK (2008) Effect of soluble and insoluble dietary fiber on embryo survival and sow performance. *Journal of Animal Science* **86** 2576–2584.

Rezende SM, Simmonds RE and Lane DA (2004) Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* **103** 1192–1202.

Rimon-Dahari N, Yerushalmi-Heinemann L, Alyagor L and Dekel N (2016) *Ovarian Folliculogenesis*.

Rizkalla SW, Prifti E, Cotillard A, Pelloux V, Rouault C, Allouche R, Laromiguière M, Kong L, Darakhshan F, Massiera F *et al.* (2012) Differential effects of macronutrient content in 2 energy-restricted diets on cardiovascular risk factors and adipose tissue cell size in moderately obese individuals: a randomized controlled trial. *The American Journal of Clinical Nutrition* **95** 49–63.

Roberts SA, Xin H, Kerr BJ, Russell JR and Bregendahl K (2007) Effects of dietary fiber and reduced crude protein on nitrogen balance and egg production in laying hens. *Poultry Science* **86** 1716–1725.

Roberts CK, Izadpanah A, Angadi SS and Barnard RJ (2013) Effects of an intensive short-term diet and exercise intervention: comparison between normal-weight and obese children. *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology* **305** 552–557.

Rodgers RJ and Irving-Rodgers HF (2010) Formation of the ovarian follicular antrum and follicular fluid. *Biology of Reproduction* **82** 1021–1029.

Rodgers HF, Lavranos TC, Vella CA and Rodgers RJ (1995) Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture. *Cell & Tissue Research* **282** 463–471.

Rodgers RJ, Irving-Rodgers HF and Russell DL (2003) Extracellular matrix of the developing ovarian follicle. *Reproduction* **126** 415–424.

Rose-Hellekant TA, Libersky-Williamson EA and Bavister BD (1998) Energy substrates and amino acids provided during in vitro maturation of bovine oocytes alter acquisition of developmental competence. *Zygote* **6** 285–294.

Ross AB, Bruce SJ, Blondel-Lubrano A, Oguey-Araymon S, Beaumont M, Bourgeois A, Nielsen-Moennoz C, Vigo M, Fay LB, Kochhar S *et al.* (2011) A whole-grain cereal-rich diet increases plasma betaine, and tends to decrease total and LDL-cholesterol compared with a refined-grain diet in healthy subjects. *British Journal of Nutrition* **105** 1492–1502.

Roura M, Catala MG, Soto-Heras S, Hammami S, Izquierdo D, Fouladi-Nashta A and Paramio MT (2017) Linoleic (LA) and linolenic (ALA) acid concentrations in follicular fluid of prepubertal goats and their effect on oocyte in vitro maturation and embryo development. *Reproduction, Fertility and Development* **Jul 6** A-K.

Ryan DP, Yaakub H, Harringtonz D and Lynch PB (1994) Follicular development during early pregnancy and the estrous cycle of the sow. *Theriogenology* **42** 623–632.

Salhab M, Tosca L, Cabau C, Papillier P, Perreau C, Dupont J, Mermillod P and Uzbekova S (2011) Kinetics of gene expression and signaling in bovine cumulus cells throughout IVM in different mediums in relation to oocyte developmental competence, cumulus apoptosis and progesterone secretion. *Theriogenology* **75** 90–104.

Sanchez-Lazo L, Brisard D, Elis S, Maillard V, Uzbekov R, Labas V, Desmarchais A, Papillier P, Monget P and Uzbekova S (2014) Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine. *Molecular Endocrinology* **28** 1502–1521.

Santonastaso M, Pucciarelli A, Costantini S, Caprio F, Sorice A, Capone F, Natella A, Iardino P, Colacurci N and Chiosi E (2017) Metabolomic profiling and biochemical evaluation of the follicular fluid of endometriosis patients. *Molecular BioSystems* **13** 1213–1222.

Sassolas A, Lagarde M, Guichardantb M, Quincy C and Dechavanne M (1983) Plasma lipoproteins and fatty acid composition after 'minipill'. *Contraception* **28** 357–368.

Sato H, Iwata H, Hayashi T, Kimura K, Kuwayama T and Monji Y (2007) The effect of glucose on the progression of the nuclear maturation of pig oocytes. *Animal Reproduction Science* **99** 299–305.

Scheideler SE and Froning GW (1996) The combined influence of dietary flaxseed variety, level, form, and storage conditions on egg production and composition among vitamin E-supplemented hens. *Poultry Science* **75** 1221–1226.

Seishima M, Bisgaier CL, Davies SL and Glickman RM (1991) Regulation of hepatic apolipoprotein synthesis in the 17α-ethinyl estradiol-treated rat. *Journal of Lipid Research* **32** 941–951.

de Seny D, Cobraiville G, Leprince P, Fillet M, Collin C, Mathieu M, Hauzeur JP, Gangji V and Malaise MG (2016) Biomarkers of inflammation and innate immunity in atrophic nonunion fracture. *Journal of Translational Medicine* **14** 1–17.

Seo H-Y, Kim M-K, Jung Y-A, Jang BK, Yoo E-K, Park K-G and Lee I-K (2013) Clusterin decreases hepatic SREBP-1c expression and lipid accumulation. *Endocrinology* **154** 1722–1730.

Sessions-Bresnahan DR and Carnevale EM (2014) The effect of equine metabolic syndrome on the ovarian follicular environment. *Journal of Animal Science* **92** 1484–1493.

Sessions-Bresnahan DR, Schauer KL, Heuberger AL and Carnevale EM (2016) Effect of obesity on the preovulatory follicle and lipid fingerprint of equine oocytes. *Biology of Reproduction* **94** 1–12.

Seto NL and Bogan RL (2015) Decreased cholesterol uptake and increased liver X receptor-mediated cholesterol efflux pathways during prostaglandin F2 alpha-induced and spontaneous luteolysis in sheep. *Biology of Reproduction* **92** 1–9.

Severino V, Malorni L, Cicatiello AE, D'Esposito V, Longobardi S, Colacurci N, Miraglia N, Sannolo N, Farina A and Chambery A (2013) An integrated approach based on multiplexed protein array and iTRAQ labeling for in-depth identification of pathways associated to IVF outcome. *PLOS ONE* **8** e77303.

Shaaker M, Rahimipour A, Nouri M, Khanaki K, Darabi M, Farzadi L, Shahnazi V and Mehdizadeh A (2012) Fatty acid composition of human follicular fluid phospholipids and fertilization rate in assisted reproductive techniques. *Iranian Biomedical Journal* **16** 162–168.

Shen L, Wang DQH, Lo CM, Tso P, Davidson WS, Woods SC and Liu M (2010) Estradiol increases the anorectic effect of central apolipoprotein A-IV. *Endocrinology* **151** 3163–3168.

Shen L, Liu Y, Wang DQH, Tso P, Woods SC and Liu M (2014) Estradiol stimulates apolipoprotein A-IV gene expression in the nucleus of the solitary tract through estrogen receptor-α. *Endocrinology* **155** 3882–3890.

Shen X, Liu X, Zhu P, Zhang Y, Wang J, Wang Y, Wang W, Liu J, Li N and Liu F (2017) Proteomic analysis of human follicular fluid associated with successful in vitro fertilization. *Reproductive Biology and Endocrinology* **15** 1–15.

Sher A and Rahman M (2000) Enterohepatic recycling of estrogen and its relevance with female fertility. *Archives of Pharmacal Research* **23** 513–517.

Shi JM, Tian XZ, Zhou GB, Wang L, Gao C, Zhu SE, Zeng SM, Tian JH and Liu GS (2009) Melatonin exists in porcine follicular fluid and improves in vitro maturation and parthenogenetic development of porcine oocytes. *Journal of Pineal Research* **47** 318–323.

Sim Y-J and Lee M-Y (2008) Identification of proteins in human follicular fluid by proteomic profiling. *Molecular and Cellular Toxicology* **4** 253–259.

Sinclair K (2008) Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development. *Reproductive BioMedicine Online* **16** 859–868.

Sinclair KD, Rutherford KMD, Wallace JM, Brameld JM, Stöger R, Alberio R, Sweetman D, Gardner DS, Perry VEA, Adam CL *et al.* (2016) Epigenetics and developmental programming of welfare and production traits in farm animals. *Reproduction, Fertility and Development* **28** 1443–1478.

Singh B, Barbe GJ and Armstrong DT (1993) Factors influencing resumption of meiotic maturation and cumulus expansion of porcine oocyte-cumulus cell complexes in vitro. *Molecular Reproduction and Development* **36** 113–119.

Siuzdak G (2004) An introduction to mass spectrometry ionization: an excerpt from the expanding role of mass spectrometry in biotechnology, 2nd edition; MCC Press: San Diego, 2005. *Journal of the Association for Laboratory Automation* **9** 50–63.

Skowronski MT, Kwon T-H and Nielsen S (2009) Immunolocalization of aquaporin 1, 5, and 9 in the female pig reproductive system. *Journal of Histochemistry & Cytochemistry* **57** 61–67.

Soares R, Franco C, Pires E, Ventosa M, Palhinhas R, Koci K, de Almeida AM and Coelho AV (2012) Mass spectrometry and animal science: Protein identification strategies and particularities of farm animal species. *Journal of Proteomics* **75** 4190–4206.

Soede NM, Langendijk P and Kemp B (2011) Reproductive Cycles in Pigs. *Animal Reproduction Science* **124** 251–258.

Song C, Yao J, Cao C, Liang X, Huang J, Han Z, Zhang Y, Qin G, Tao C, Li C *et al.* (2016) PPARγ is regulated by miR-27b-3p negatively and plays an important role in porcine oocyte maturation. *Biochemical and Biophysical Research Communications* **479** 224–230.

Souza da Silva C, van den Borne JJGC, Gerrits WJJ, Kemp B and Bolhuis JE (2012) Effects of dietary fibers with different physicochemical properties on feeding motivation in adult female pigs. *Physiology and Behavior* **107** 218–230.

Sriperumbudur R, Zorrilla L and Gadsby JE (2010) Transforming growth factor-β (TGFβ) and its signaling components in peri-ovulatory pig follicles. *Animal Reproduction Science* **120** 84–94.

Srivastava RAK, Kitchens RT and Schonfeld G (1994) Regulation of the apolipoprotein AIV gene expression by estrogen differs in rat and mouse. *European Journal of Biochemistry* **222** 507–514.

Standaert FE, Zamora CS and Chew BP (1991) Quantitative and qualitative changes in blood leukocytes in the porcine ovary. *American Journal of Reproductive Immunology* **25** 163–168.

Stanton PG, Foo CFH, Rainczuk A, Stephens AN, Condina M, O'Donnell L, Weidner W, Ishikawa T, Cruickshanks L, Smith LB *et al.* (2016) Mapping the testicular interstitial fluid proteome from normal rats. *Proteomics* **16** 2391–2402.

Stone SL, Pomerantz SH, Schwartz-Kripner A and Channing CP (1978) Inhibitor of oocyte maturation from porcine follicular fluid: further purification and evidence for reversible action. *Biology of Reproduction* **19** 585–592.

Sturmey RG and Leese HJ (2003) Energy metabolism in pig oocytes and early embryos. *Reproduction* **126** 197–204.

Sturmey RG, Reis A, Leese HJ and McEvoy TG (2009) Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reproduction in Domestic Animals* **44** 50–58.

Suchanek E, Mujkic-Klaric A, Grizelj V, Simunic V and Kopjar B (1990) Protein concentration in pre ovulatory follicular fluid related to ovarian stimulation. *International Journal of Gynecology and Obstetrics* **32** 53–59.

Sun Y-L, Ping Z-G, Li C-J, Sun Y-L, Yi K-L, Chen L, Li X-Y, Wang X-L and Zhou X (2011) Comparative proteomic analysis of follicular fluids from normal and cystic follicles in sows. *Reproduction in Domestic Animals* **46** 889–895.

Sun HQ, Zhou YF, Tan CQ, Zheng LF, Peng J and Jiang SW (2014) Effects of konjac flour inclusion in gestation diets on the nutrient digestibility, lactation feed intake and reproductive performance of sows. *Animal* **8** 1089–1094.

Surampudi P, Enkhmaa B, Anuurad E and Berglund L (2016) Lipid lowering with soluble dietary fiber. *Current Atherosclerosis Reports* **18** 1–13.

Sutton-McDowall ML, Gilchrist RB and Thompson JG (2010) The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* **139** 685–695.

Sutton ML, Gilchrist RB and Thompson JG (2003) Effect of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update* **9** 35–48.

Tacoma R, Fields J, Ebenstein DB, Lam Y-W and Greenwood SL (2016) Comparative proteomics dataset of skimmed milk samples from Holstein and

Jersey dairy cattle. *Data in Brief* **6** 843–846.

Tacoma R, Gelsinger SL, Lam YW, Scuderi RA, Ebenstein DB, Heinrichs AJ and Greenwood SL (2017a) Exploration of the bovine colostrum proteome and effects of heat treatment time on colostrum protein profile. *Journal of Dairy Science* **100** 1–10.

Tacoma R, Fields J, Ebenstein DB, Lam Y and Greenwood SL (2017b) Ratio of dietary rumen degradable protein to rumen undegradable protein affects nitrogen partitioning but does not affect the bovine milk proteome produced by mid-lactation Holstein dairy cows. *Journal of Dairy Science* **100** 7246–7261.

Tao J, Zhao G, Zhao X, Li F, Wu X, Hu J and Zhang Y (2014) Proteomic analysis of the follicular fluid of Tianzhu white yak during diestrus. *International Journal of Molecular Sciences* **15** 4481–4491.

Tao C, Li J, Chen B, Chi D, Zeng Y and Liu H (2017) Genome-scale identification of nucleosome organization by using 1000 porcine oocytes at different developmental stages. *PLOS ONE* **12** 1–14.

Tatemoto H, Sakurai N and Muto N (2000) Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. *Biology of Reproduction* **63** 805–810.

Tatemoto H, Muto N, Sunagawa I, Shinjo A and Nakada T (2004) Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. *Biology of Reproduction* **71** 1150–1157.

Thomas FH, Ethier J-F, Shimasaki S and Vanderhyden BC (2005) Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. *Endocrinology* **146** 941–949.

Timms JF, Arslan-Low E, Kabir M, Worthington J, Camuzeaux S, Sinclair J, Szaub J, Afrough B, Podust VN, Fourkala E *et al.* (2014) Discovery of serum biomarkers of ovarian cancer using complementary proteomic profiling strategies. *Proteomics. Clinical Applications* **8** 982–993.

Toms D, Tsoi S, Dobrinsky J, Dyck MK and Li J (2014) The effects of glial cell line-derived neurotrophic factor on the in vitro matured porcine oocyte transcriptome. *Molecular Reproduction and Development* **81** 217–229.

Tsafriri A (1995) Ovulation as a tissue remodelling process. In *Tissue Renin-Angiotensin Systems. Advances in Experimental Medicine and Biology*, 377th ed, pp 121–140. Eds M A.K and R M.K. Boston, MA: Springer.

Tsang BK, Ainsworth L, Downey BR and Armstrong DT (1979) Pre-ovulatory changes in cyclic AMP and prostaglandin concentrations in follicular fluid of gilts. *Prostaglandins* **17** 141–148.

Tumanov S, Bulusu V and Kamphorst JJ (2015) Analysis of fatty acid metabolism using stable isotope tracers and mass spectrometry. *Methods in Enzymology* **561** 197–217.

Lo Turco EG, Cordeiro FB, Lopes PH de C, Gozzo FC, Pilau EJ, Soler TB, da Silva BF, Del Giudice PT, Bertolla RP, Fraietta R *et al.* (2013) Proteomic analysis of follicular fluid from women with and without endometriosis: New therapeutic targets and biomarkers. *Molecular Reproduction and Development* **80** 441–450.

Twigt J, Steegers-Theunissen RP, Bezstarosti K and Demmers JAA (2012) Proteomic analysis of the microenvironment of developing oocytes. *Proteomics* **12** 1463–1471.

Twigt JM, Bezstarosti K, Demmers J, Lindemans J, Laven JSE and Steegers-Theunissen RP (2015) Preconception folic acid use influences the follicle fluid proteome. *European Journal of Clinical Investigation* **45** 833–841.

Valckx SDM, Arias-Alvarez M, Pauw I De, Fievez V, Vlaeminck B, Fransen E, Bols PEJ and Leroy JLMR (2014) Fatty acid composition of the follicular fluid of normal weight, overweight and obese women undergoing assisted reproductive treatment: a descriptive cross-sectional study. *Reproductive Biology and Endocrinology* **12** 1–11.

Valero-Muñoz M, Martín-Fernández B, Ballesteros S, Lahera V and de las Heras N (2014) Carob pod insoluble fiber exerts anti-atherosclerotic effects in rabbits through sirtuin-1 and peroxisome proliferator-activated receptor-γ coactivator-1α. *The Journal of Nutrition* **144** 1378–1384.

Valsangkar D and Downs SM (2013) A requirement for fatty acid oxidation in the hormone-induced meiotic maturation of mouse oocytes. *Biology of Reproduction* **89** 1–9.

Venkatesan N, Devaraj SN and Devaraj H (2007) A fibre cocktail of fenugreek, guar gum and wheat bran reduces oxidative modification of LDL induced by an atherogenic diet in rats. *Molecular and Cellular Biochemistry* **294** 145–153.

Venter C, Nel C, Vorster H, Jerling J, Oosthuizen W, Veldman F, Kellerman J, Smuts C, Vermaak W, van der Nest D *et al.* (1997) Soluble-fibre concentrate lowers plasminogen activator inhibitor-1 in baboons (Papio ursinus). *British Journal of Nutrition* **78** 625–637.

Vernon RG (1980) Comparative aspects of lipid metabolism in monogastric, pre-ruminant and ruminating animals. *Biochemical Society Transactions* **8** 291–292.

Veum TL, Crenshaw JD, Crenshaw TD, Cromwell GL, Easter RA, Ewan RC, Nelssen JL, Miller ER, Pettigrew JE and Ellersieck MR (2009) The addition of ground wheat straw as a fiber source in the gestation diet of sows and the effect on sow and litter performance for three successive parities. *Journal of Animal Science* **87** 1003–1012.

Vidotti D, Schnabel B, Secco M, Cristovam P, Borra R and da Silva I (2015) Genetic analysis and cAMP measurement: comparison between lean and obese anovulating mice. *Revista Brasileira de Ginecologia e Obstetrícia* **37** 186–191.

Wallace M, Cottell E, Gibney MJ, Mcauliffe FM, Wingfield M and Brennan L (2012) An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. *Fertility and Sterility* **97** 1078–1084.

Wang JM, Irwin RW and Brinton RD (2006) Activation of estrogen receptor α increases and estrogen receptor β decreases apolipoprotein E expression in hippocampus in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **103** 16983–16988.

Wang Y, Rogers PM, Su C, Varga G, Stayrook KR and Burris TP (2008) Regulation of cholesterologenesis by the oxysterol receptor, LXRα. *Journal of Biological Chemistry* **283** 26332–26339.

Wang XL, Wu Y, Tan L Bin, Tian Z, Liu JH, Zhu DS and Zeng SM (2012) Follicle-stimulating hormone regulates pro-apoptotic protein Bcl-2-interacting mediator of cell death-extra long (BimEL)-induced porcine granulosa cell apoptosis. *Journal of Biological Chemistry* **287** 10166–10177.

Wang X-L, Wang K, Zhao S, Wu Y, Gao H and Zeng S-M (2013a) Oocyte-secreted growth differentiation factor 9 inhibits BCL-2-interacting mediator of cell death-extra long expression in porcine cumulus cell. *Biology of Reproduction* **89** 1–9.

Wang W, Zhang W, Liu J, Sun Y, Li Y, Li H, Xiao S and Shen X (2013b) Metabolomic changes in follicular fluid induced by soy isoflavones administered to rats from weaning until sexual maturity. *Toxicology and Applied Pharmacology* **269** 280–289.

Warzych E, Cieslak A, Pawlak P, Renska N, Pers-Kamczyc E and Lechniak D (2011) Maternal nutrition affects the composition of follicular fluid and transcript content in gilt oocytes. *Veterinarni Medicina* **56** 156–167.

Weaver AC, Kelly JM, Kind KL, Gatford KL, Kennaway DJ, Herde PJ and Van Wettere WHEJ (2013) Oocyte maturation and embryo survival in nulliparous female pigs (gilts) is improved by feeding a lupin-based high-fibre diet. *Reproduction, Fertility and Development* **25** 1216–1223.

Wei J, Shi Y, Zhang X, Feng Y, Luo G, Zhang J, Mu Q, Tang Y, Yu Y, Pan L *et al.* (2011) Estrogen upregulates hepatic apolipoprotein M expression via the estrogen receptor. *Biochimica et Biophysica Acta* **1811** 1146–1151.

Wei J, Yu Y, Luo G, Feng Y, Shi Y, Zhang J, Mu Q, Yu M, Pan L, Berggren-Söderlund M *et al.* (2017) 17β-estradiol regulates the expression of apolipoprotein M through estrogen receptor α-specific binding motif in its promoter. *Lipids in Health and Disease* **16** 1–8.

Wiel DFM van de, Bar-Ami S, Tsafriri A and de Jong FH (1983) Oocyte maturation inhibitor, inhibin and steroid concentrations in porcine follicular fluid at various stages of the oestrous cycle. *Journal of Reproduction and Fertility* **68** 247–252.

Wójcik-Gładysz A and Polkowska J (2006) Neuropeptide Y - a neuromodulatory link between nutrition and reproduction at the central nervous system level. *Reproductive Biology* **6** 21–28.

Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC and Sinclair KD (2010) Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction* **139** 57–69.

Wrana JL and Attisano L (2000) The Smad pathway. *Cytokine and Growth Factor Reviews* **11** 5–13.

Wu MF, Huang WT, Tsay C, Hsu HF, Liu BT, Chiou CM, Yen SC, Cheng SP and Ju JC (2002) The stage-dependent inhibitory effect of porcine follicular cells on the development of preantral follicles. *Animal Reproduction Science* **73** 73–88.

Wu YT, Wu Y, Zhang JY, Hou NN, Liu AX, Pan JX, Lu JY, Sheng JZ and Huang HF (2015) Preliminary proteomic analysis on the alterations in follicular fluid proteins from women undergoing natural cycles or controlled ovarian hyperstimulation. *Journal of Assisted Reproduction and Genetics* **32** 417–427.

Wu Y, Lin J, Han B, Wang L, Chen Y, Liu M and Huang J (2017) Proteomic profiling of follicle fluids after superstimulation in one-month-old lambs. *Reproduction in Domestic Animals* 1–9.

Wunder DM, Kretschmer R and Bersinger NA (2005) Concentrations of leptin and C-reactive protein in serum and follicular fluid during assisted reproductive cycles. *Human Reproduction* **20** 1266–1271.

Xia L, Zhao X, Sun Y, Hong Y, Gao Y and Hu S (2014) Metabolomic profiling of human follicular fluid from patients with repeated failure of in vitro fertilization using gas chromatography/ mass spectrometry. *International Journal of Clinical and Experimental Pathology* **7** 7220–7229.

Xie S, Broermann DM, Nephew KP, Ottobre JS, Day ML and Pope WF (1990) Changes in follicular endocrinology during final maturation of porcine oocytes. *Domestic Animal Endocrinology* **7** 75–82.

Xie D, Lau SH, Sham JST, Wu Q, Fang Y, Liang L, Che L, Zeng Y and Guan X-Y (2005) Up-regulated expression of cytoplasmic clusterin in human ovarian carcinoma. *Cancer* **103** 277–283.

Xingshen S, Kuizhong Y, Suofeng M, Yi Y, Zou Y and Tan J (2002) Sources of cumulus expansion enabling factor (CEEF) in porcine follicles. *Chinese Science*

Bulletin **47** 1619–1622.

Yanagishita M, Rodbard D and Hascall VC (1978) Isolation and characterization of proteoglycans from porcine ovarian follicular fluid. *Journal of Biological Chemistry* **254** 911–920.

Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ and Robker RL (2012) Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertility and Sterility* **97** 1438–1443.

Yao JK, Chang SCS, Ryan RJ and Dyck PJ (1978) A model for studying LCAT reaction: in vitro cholesterol esterification in pig ovarian follicular fluid. *Lipids* **13** 225–231.

Yao JK, Ryan RJ and Dyck PJ (1980) The Porcine Ovarian Follicle. VI. Comparison of fatty acid composition of serum and follicular fluid at different developmental stages. *Biology of Reproduction* **22** 141–147.

Yde CC, Bertram HC, Theil PK and Knudsen KEB (2011) Effects of high dietary fibre diets formulated from by-products from vegetable and agricultural industries on plasma metabolites in gestating sows. *Archives of Animal Nutrition* **65** 460–476.

Yde CC, Westerhuis JA, Bertram HC and Knudsen KEB (2012) Application of NMR-based metabonomics suggests a relationship between betaine absorption and elevated creatine plasma concentrations in catheterised sows. *The British Journal of Nutrition* **107** 1603–1615.

Ye M, Pan Y, Cheng K and Zou H (2014) Protein digestion priority is independent of protein abundances. *Nature Methods* **11** 220–222.

Yi D, Zeng S and Guo Y (2012) A diet rich in n-3 polyunsaturated fatty acids reduced prostaglandin biosynthesis, ovulation rate, and litter size in mice. *Theriogenology* **78** 28–38.

Yokoo M, Kimura N, Abe H and Sato E (2008) Influence of hyaluronan accumulation during cumulus expansion on in vitro porcine oocyte maturation. *Zygote* **16** 309–314.

Yoshida M, Ishizaki Y and Kawagishi H (1990) Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. *Journal of Reproduction and Fertility* **88** 1–8.

Yoshida M, Ishizaki Y, Kawagishi H, Bamba K and Kojima Y (1992) Effects of pig follicular fluid on maturation of pig oocytes in vitro and on their subsequent fertilizing and developmental capacity in vitro. *Journal of Reproduction and Fertility* **95** 481–488.

Yuan B, Liang S, Kwon JW, Jin YX, Park SH, Wang HY, Sun TY, Zhang JB

and Kim NH (2016) The role of glucose metabolism on porcine oocyte cytoplasmic maturation and its possible mechanisms. *PLOS ONE* **11** 1–15.

Zachut M, Arieli A, Lehrer H, Argov N and Moallem U (2008) Dietary unsaturated fatty acids influence preovulatory follicle characteristics in dairy cows. *Reproduction* **135** 683–692.

Zachut M, Dekel I, Lehrer H, Arieli A, Arav A, Livshitz L, Yakoby S and Moallem U (2010) Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. *Journal of Dairy Science* **93** 529–545.

Zachut M, Arieli A and Moallem U (2011) Incorporation of dietary n-3 fatty acids into ovarian compartments in dairy cows and the effects on hormonal and behavioral patterns around estrus. *Reproduction* **141** 833–840.

Zamah AM, Hassis ME, Albertolle ME and Williams KE (2015) Proteomic analysis of human follicular fluid from fertile women. *Clinical Proteomics* **12** 1–12.

Zhang X-Y, Jiao G-Q, Hurtig M, Dong X, Zheng L, Luo G, Nilsson-Ehle P, Ye Q and Xu N (2004) Expression pattern of apolipoprotein M during mouse and human embryogenesis. *Acta Histochemica* **106** 123–128.

Zhang X, Zhu Z, Luo G, Zheng L, Nilsson-Ehle P and Xu N (2008) Liver X receptor agonist downregulates hepatic apoM expression in vivo and in vitro. *Biochemical and Biophysical Research Communications* **371** 114–117.

Zhang T, Chen S, Syed I, Ståhlman M, Kolar MJ, Homan EA, Chu Q, Smith U, Borén J, Kahn BB *et al.* (2016) A LC-MS-based workflow for measurement of branched fatty acid esters of hydroxy fatty acids. *Nature Protocols* **11** 747–763.

Zhang Y, Liu L, Yin T, Yang J and Xiong C (2017) Follicular metabolic changes and effects on oocyte quality in polycystic ovary syndrome patients. *Oncotarget* **8** 80472–80480.

Zhao C and Dahlman-Wright K (2010) Liver X receptor in cholesterol metabolism. *Journal of Endocrinology* **204** 233–240.

Zhao H, Zhao Y, Li T, Li M, Li J, Li R, Liu P, Yu Y and Qiao J (2015) Metabolism alteration in follicular niche: The nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. *Free Radical Biology and Medicine* **86** 295–307.

Zhu G, Guo B, Pan D, Mu Y and Feng S (2008) Expression of bone morphogenetic proteins and receptors in porcine cumulus-oocyte complexes during in vitro maturation. *Animal Reproduction Science* **104** 275–283.

Zhu C, Di D, Zhang X, Luo G, Wang Z, Wei J, Shi Y, Berggren-Söderlund M, Nilsson-Ehle P and Xu N (2011) TO901317 regulating apolipoprotein M

expression mediates via the farnesoid X receptor pathway in Caco-2 cells. *Lipids in Health and Disease* **10** 1–6.

Ziecik AJ, Przygrodzka E and Kaczmarek MM (2017) Corpus luteum regression and early pregnancy maintenance in pigs. In *The Life Cycle of the Corpus Luteum*, pp 227–248.

Zolotarjova N, Martosella J, Nicol G, Bailey J, Boyes BE and Barrett WC (2005) Differences among techniques for high-abundant protein depletion. *Proteomics* **5** 3304–3313.

Zwain I and Amato P (2000) Clusterin protects granulosa cells from apoptotic cell death during follicular atresia. *Experimental Cell Research* **257** 101–110.

Appendices

<table>
<tr><th>Animal number</th><th>Diet given</th><th>Blastocyst following IVF?</th><th>Used in proteomics/western blots?</th><th>Used in metabolomics?</th></tr>
<tr><td rowspan="10">8370
8622
8647
8681
8451
8418
8614
8471
8431
8400
8363</td><td rowspan="10">Control</td><td rowspan="5">No</td><td rowspan="3">Yes;
For All-C and C-No experimental pools</td><td>Yes</td></tr>
<tr><td rowspan="4">No</td></tr>
<tr><td rowspan="2">No</td></tr>
<tr><td rowspan="5">Yes</td><td rowspan="3">Yes;
For All-C and C-Bl experimental pools</td><td>Yes</td></tr>
<tr><td rowspan="4">No</td></tr>
<tr><td rowspan="2">No</td></tr>
<tr><td rowspan="10">8645
8617
8612
8686
8424
8368
8675
8652
8648
8476
8457
8454
8401
8374</td><td rowspan="10">High Fibre</td><td rowspan="6">No</td><td rowspan="3">Yes;
For All-HF and HF-No experimental pools</td><td>Yes</td></tr>
<tr><td rowspan="5">No</td></tr>
<tr><td rowspan="3">No</td></tr>
<tr><td rowspan="4">Yes</td><td rowspan="3">Yes;
For All-HF and HF-Bl experimental pools</td><td>Yes</td></tr>
<tr><td rowspan="3">No</td></tr>
<tr><td>188
8426
8644
8419
8430
8455</td></tr>
</table>

Appendix 1. Porcine follicular fluid samples used in the experiments.

Codes for the animals that were fed either a high fibre diet or a control diet and which animals' pFF samples were used in either the proteomic and/or metabolomic analyses.

Animal ID	Group	PFF E2 concentration (ng/mL)	Cleavage rates (%)	Blastocyst rate (%)	Blastocyst cell number	Mean follicle size (mm)	Mean pFF volume per follicle (µl)
8370	C-No	89	N/A	N/A	N/A	6	80
8622		172	N/A	N/A	N/A	6	120
8647		76	N/A	N/A	N/A	7	140
8377	C-Bl	111	19	66	22, 23	6	100
8682		105	60	22	25, 31	6	85
8687		157	57	12.5	39	7	140
8645	HF-No	122	N/A	N/A	N/A	7	100
8617		164	N/A	N/A	N/A	7	100
8612		270	N/A	N/A	N/A	7	120
188	HF-Bl	166	33	20	33	7	110
8426		164	73	28	49, 46, 43	7	110
8644		144	29	75	34, 32, 37	7	120

Appendix 2. Reproductive parameters associated with the porcine follicular fluid of animals used in the metabolomic analysis.

Reproductive parameters were recorded by Dr Elizabeth M. Ferguson (Ashworth *et al.*, 2008) analysis. Some animals have multiple cleavage rates, blastocyst rates and blastocyst cell number values as they had more than one oocyte that successfully fertilised. Abbreviations: E_2 conc. = oestradiol concentration; C-No = control-fed pigs whose oocytes did not produce a blastocyst; C-Bl = control-fed pigs whose oocytes produced a blastocyst; HF-No = high fibre-fed pigs whose oocytes did not produce a blastocyst; HF-Bl = high fibre-fed pigs whose oocytes produced a blastocyst.

Ingredient	Percentage composition (%)	
	Control	**High Fibre**
Sugar Beet Pulp	0.00	49.96
Barley	63.00	18.01
Wheat	20.00	17.32
Hipro Soya	11.00	9.52
Fishmeal	2.50	2.16
Soya Oil	1.00	0.87
Dicalcium Phosphate	1.00	1.67
Salt	0.40	0.35
Limestone	0.93	0.00
Trouw S126	0.17	0.15
Total Percentage (%)	100.00	100.00

Appendix 3. Composition of control barley-based diet and high fibre diet supplemented with 50% unmolassed sugar beet pulp.

The diets were isocaloric formulated by Norvite and were used in the IVF experiment that generated the pFF samples used in this study (Ashworth *et al.*, 2008).

	Mean Concentration (µg/mL) ± Standard Deviation (µg/mL) Inter-Assay Coefficient of Variation (%)								
Animal	**Arachidic**	**Palmitoleic**	**Oleic**	**Linoleic**	**Erucic**	**Arachidonic**	**DGLA**	**Adrenic**	**DPA**
C-No-1	0.6 ± 0.1 16.71	25.4 ± na na	74.2 ± 18.5 24.92	82.4 ± 6.4 7.72	0.1 ± 0.0 31.6	59.9 ± 5.7 9.47	4.2 ± 1.3 30.29	47.3 ± 3.5 7.48	14.8 ± 2.9 19.49
C-No-2	0.5 ± 0.2 31.20	23.7 ± 0.8 3.54	77.8 ± 2.7 3.44	101.0 ± 3.7 3.61	0.1 ± 0.1 47.60	39.8 ± 7.2 18.17	2.2 ± 0.6 25.27	22.3 ± 2.6 11.23	9.4 ± 1.9 20.03
C-No-3	0.5 ± 0.1 21.66	25.0 ± 3.2 12.91	94.3 ± 4.4 4.67	70.0 ± 3.52 5.02	0.15 ± 0.0 25.74	55.5 ± 10.3 18.49	3.2 ± 1.0 30.02	39.8 ± 5.5 13.9	13.0 ± 2.3 17.9
C-No-4	0.8 ± na na	22.4 ± 1.9 8.66	89.3 ± 30.1 33.65	74.1 ± 6.4 8.68	0.2 ± na na	39.5 ± 2.1 5.23	3.1 ± 0.9 29.85	34.5 ± 4.1 11.92	11.4 ± 1.8 15.39
C-No-5	0.4 ± na na	23.8 ± 3.1 12.86	77.7 ± 4.6 5.86	82.2 ± 4.4 5.36	2.8 ± 5.6 203.25	43.6 ± 3.3 7.64	3.2 ± 0.8 25.30	40.8 ± 8.2 20.13	9.7 ± 0.9 8.92
C-No-6	0.3 ± 0.0 1.00	30.9 ± 7.0 22.78	98.2 ± 55.7 56.66	114. 9 ± 13.0 11.33	0.3 ± 0.3 112.61	59.3 ± 2.9 4.93	4.2 ± 1.2 29.70	48.3 ± 10.9 22.66	15.0 ± 3.2 21.54
C-Bl-1	0.5 ± 0.3 59.73	40.2 ± na na	101.5 ± 27.9 27.43	145.5 ± 10.4 7.18	0.2 ± 0.1 35.69	100.1 ± 10.0 10.03	4.9 ± 1.0 20.10	80.1 ± 1.3 1.59	15.9 ± 2.4 14.82
C-Bl-2	0.4 ± 0.1 17.53	30.9 ± 0.4 1.22	121.7 ± 7.8 6.41	117.7 ± 0.6 0.52	0.1 ± 0.0 7.22	58.1 ± 23.0 39.48	4.8 ± 0.9 19.30	36.7 ± 4.2 11.50	14.5 ± 2.1 14.31
C-Bl-3	0.5 ± 0.4 72.61	21.5 ± 0.6 2.83	78.0 ± 10.9 13.99	81.5 ± 5.3 6.45	0.2 ± 0.0 14.84	57.2 ± 6.8 11.80	3.5 ± 1.1 31.55	43.0 ± 5.3 12.25	10.9 ± 3.1 27.94
C-Bl-4	0.7 ± na na	29.5 ± 3.8 13.00	100.1 ± 36.6 36.53	104.3 ± 11.3 10.83	5.2 ± 10.6 202.44	55.3 ± 0.0 0.05	4.3 ± 0.7 17.31	48.5 ± 0.0 0.00	13.0 ± 2.0 15.00
C-Bl-5	0.5 ± 0.0 4.96	21.7 ± 0.5 2.26	66.9 ± 2.0 3.03	62.7 ± 1.8 2.85	0.1 ± na na	28.3 ± 11.5 40.66	3.2 ± 0.9 28.71	21.2 ± 4.3 20.25	9.0 ± 1.1 12.37
C-Bl-6	0.7 ± 0.3 37.48	24.3 ± 2.9 11.75	75.0 ± 27.1 36.13	99.5 ± 8.5 8.54	3.9 ± 5.2 136.16	41.7 ± 6.3 15.11	3.3 ± 0.4 13.32	44.3 ± 0.2 0.33	9.4 ± 0.3 3.21

Appendix 4. Mean concentration, standard deviation and inter-assay coefficient of variation for each fatty acid in follicular fluid samples from control-fed pigs.

Mean concentrations (µg/mL) and standard deviations (µg/mL) and inter-assay coefficient of variation (%) for each fatty acid in follicular fluid from pigs fed the control diet whose oocytes formed a blastocyst following *in vitro* fertilisation (C-Bl) and control-fed pigs whose oocytes did not have a blastocyst (C-No). Samples 1-3 were assayed in batches A and C whilst samples 4-6 were assayed in batches B and D. Abbreviations: DGLA = dihomo-γ-linolenic acid; DPA = docosapentaenoic acid.

	Mean Concentration (µg/mL) ± Standard Deviation (µg/mL) [Inter-Assay Coefficient of Variation (%)]								
Animal	**Arachidic**	**Palmitoleic**	**Oleic**	**Linoleic**	**Erucic**	**Arachidonic**	**DGLA**	**Adrenic**	**DPA**
HF-No-1	0.5 ± 0.2 38.26	28.7 ± na na	93.9 ± 22.4 23.85	85.6 ± 6.7 7.80	0.2 ± 0.1 40.74	51.7 ± 4.3 8.25	3.8 ± 1.5 40.21	37.7 ± 5.5 14.57	11.5 ± 3.2 27.42
HF-No-2	0.6 ± 0.2 32.13	24.0 ± 3.0 12.67	98.3 ± 5.7 5.76	69.2 ± 7.6 10.96	0.2 ± 0.1 33.13	51.9 ± 8.3 15.98	3.4 ± 1.0 30.12	29.2 ± 2.7 9.24	11.5 ± 1.0 8.33
HF-No-3	0.3 ± 0.2 63.75	46.0 ± na na	116.5 ± 31.7 27.18	117.6 ± 25.9 22.01	0.1 ± 0.0 20.23	92.2 ± 25.6 27.80	4.8 ± 2.0 42.43	46.7 ± 7.7 16.56	12.8 ± 3.6 28.31
HF-No-4	1.3 ± na na	42.2 ± 11.3 26.82	116.9 ± 66.1 56.55	107.9 ± 5.8 5.39	0.2 ± 0.0 11.75	61.7 ± 11.9 19.32	5.3 ± 1.9 35.66	57.4 ± 5.2 9.06	18.0 ± 4.6 25.63
HF-No-5	0.9 ± na na	20.4 ± 3.0 14.50	60.5 ± 7.0 11.53	53.7 ± 1.5 2.78	0.2 ± na na	17.2 ± 4.7 27.10	2.7 ± 0.6 22.10	22.8 ± 1.6 6.92	8.1 ± 0.1 0.71
HF-No-6	0.3 ± 0.4 43.54	23.2 ± 1.0 4.49	85.1 ± 45.9 53.90	68.4 ± 2.1 3.04	4.0 ± 5.3 134.70	58.8 ± 9.4 16.03	4.3 ± 0.9 20.91	76.2 ± 4.7 6.22	17.2 ± 4.7 27.09
HF-Bl-1	0.7 ± 0.3 48.26	27.01 ± na na	94.07 ± 54.7 58.13	90.73 ± 16.8 18.54	0.17 ± 0.1 29.77	62.54 ± 31.6 50.52	4.84 ± 0.4 8.95	57.97 ± 3.6 6.13	15.36 ± 0.1 0.83
HF-Bl-2	0.7 ± 0.3 49.83	23.90 ± na na	108.66 ± 23.5 21.65	70.96 ± 1.6 2.29	0.17 ± 0.0 14.76	40.56 ± 15.6 38.45	3.64 ± 0.6 16.60	38.62 ± 3.5 9.06	11.81 ± 2.4 20.08
HF-Bl-3	0.6 ± 0.3 50.94	22.68 ± 0.6 2.62	91.26 ± 15.7 17.15	72.40 ± 5.7 7.85	0.16 ± 0.0 16.20	50.90 ± 2.8 5.54	3.72 ± 1.0 26.53	29.16 ± 3.2 10.85	12.85 ± 1.8 14.08
HF-Bl-4	1.5 ± na na	25.22 ± 3.3 13.09	92.91 ± 20.7 22.25	72.44 ± 0.4 0.50	0.32 ± na na	47.76 ± 5.4 11.19	3.82 ± 1.4 35.58	38.73 ± 1.4 3.69	16.44 ± 2.9 17.72
HF-Bl-5	1.1 ± 0.0 2.04	21.95 ± 3.0 13.46	79.58 ± 29.4 36.92	50.27 ± 0.3 0.51	0.17 ± na na	26.21 ± 2.1 7.89	2.99 ± 0.5 17.21	29.94 ± 1.7 5.67	8.72 ± 0.1 1.42
HF-Bl-6	0.5 ± 0.5 109.67	15.91 ± 2.1 13.33	57.38 ± 11.2 19.58	49.09 ± 3.9 7.94	4.45 ± 6.1 136.72	31.65 ± 1.3 4.05	2.89 ± 0.8 27.95	37.97 ± 4.9 12.85	10.30 ± 0.4 4.23

Appendix 5. Mean concentration, standard deviation and inter-assay coefficient of variation for each fatty acid in follicular fluid samples from high fibre-fed pigs.

Mean concentrations (µg/mL) and standard deviations (µg/mL) and inter-assay coefficient of variation (%) for each fatty acid in follicular fluid from pigs fed the high fibre diet whose oocytes formed a blastocyst following *in vitro* fertilisation (C-Bl) and high fibre-fed pigs whose oocytes did not have a blastocyst (C-No). Samples 1-3 were assayed in batches A and C whilst samples 4-6 were assayed in batches B and D. Abbreviations: DGLA = dihomo-γ-linolenic acid; DPA = docosapentaenoic acid.

Fatty Acids	Intra-Assay Coefficient of Variation (%)			
	A	B	C	D
Arachidic acid	16.41	48.48	50.40	51.57
Palmitoleic acid	na	2.72	4.71	5.06
Oleic acid	7.03	18.98	40.10	24.83
Linoleic acid	10.30	4.91	6.41	7.00
Erucic acid	7.65	21.81	6.16	62.94
Arachidonic acid	10.36	23.66	31.25	16.08
Dihomo-γ-linolenic acid (DGLA)	10.33	3.79	4.08	4.56
Adrenic acid	4.02	4.74	4.28	8.21
Docosapentaenoic acid (DPA)	6.74	5.98	3.78	9.31

Appendix 6. Intra-assay coefficient (%) of variation of each fatty acid within each batch analysis.